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TITLE: INTERACTION BETWEEN DIETARY FACTORS AND INFLAMMATION IN
PROSTATE CARCINOGENESIS

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14. ABSTRACT We discovered that PhIP treatment causes widespread epithelial atrophy in the rat ventral prostate that precedes the development of PIN. This data supports the contention that the PhIP-treated rat model of prostate cancer is a valid model for studying the development of PIA, PIN and early carcinoma. The results raise the novel hypothesis that suggests that the extensive atrophy that is seen in men with prostate cancer, and which has been associated with PIN and prostate cancer, may be caused by ingestion of cooked meats. This result will open up new areas of investigation into this question. For example, it would be highly feasible and reasonable to ask in a pathological-epidemiological study to determine whether the extent of prostate atrophy correlated with dietary intake of PhIP. Also, broccoli and celebrex can decrease mutations in the rat prostate induced by PhIP indicating they may be effective chemopreventative agents. Preliminary analyzes indicates that short term treatment with PhIP (4 weeks) combined with viral infection does not increase PIN or prostate cancer lesions in Fisher Rats suggesting that longer term treatment is necessary. Additional studies to complete this work are ongoing using funding sources outside of the DOD. In addition we have successfully contributed to the further instruction of an outstanding Urologist-In-Training in many aspects of prostate cancer research.

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Prostate cancer, PhIP, chemoprevention

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INTRODUCTION

Overview

One of the overarching goals of our laboratory is to understand precisely why prostate cancer is so common in men in western cultures, but is very so rare in men from East and Southeast Asia. Armed with such insights, we wish to develop novel methods to prevent this disease. The preponderance of epidemiological data suggests that environmental factors are involved in this geographical difference and that the most likely environmental factor is the diet. One of the key differences in the diet between those in western cultures and those in Eastern and Southeast Asia is that westerners consume much more meat.

Over the last decade, our laboratory has been particularly interested in the role of inflammation in prostate cancer development and we have developed a novel model of prostate carcinogenesis at the cellular and molecular level. This model suggests that chronic and acute inflammation injure the prostatic epithelium which results in regenerative lesions that we refer to as proliferative inflammatory atrophy (PIA).

In this proposal we address a novel hypothesis that indicates that a combination of a dietary carcinogens, that are produced when cooking meats at high temperature, and inflammation conspire together to cause initiation and promotion of prostate epithelial cell neoplastic transformation. The work completed during this funding period that was supported by this award has added significant new insights and discoveries that will help us to ultimately reach our overarching goal.

This report will serve as the final report for this award. Accomplishments for years one and year two will be summarized from previous reports and new accomplishments that we have made during year 3, which we consider substantial and significant, will be highlighted in yellow.

Specific Questions Addressed in this Proposal

Several studies have demonstrated that the consumption of heterocyclic amine compounds, which are produced from the charring or over-cooking of meats, like 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), is associated with prostate cancer. Interestingly, new evidence from the fields of population and genetic epidemiology, molecular pathology and molecular genetics has brought attention to the possible role of inflammation in prostate carcinogenesis. Utilizing a novel method of inciting rodent prostate inflammation developed by our laboratory, in an established animal model of PhIP-induced prostate cancer, we found that viral-induced prostate inflammation can increase the frequency of mutations in the prostate, and, when incited in conjunction with dietary exposure to PhIP, can further increase prostate DNA mutation frequency and lead to chronic prostate inflammation. In this award we sought to determine if inflammation can augment carcinogenesis is this rat model and if certain dietary chemo preventative agents can prevent cancer in this model.

BODY

The Specific Aims of this proposal were the following:

Aim (1) Evaluation of candidate dietary prostate cancer chemopreventive agents (broccoli tea, soy protein, vitamin E, lycopene) for their ability to alter DNA mutagenesis and chronic prostate inflammation in a novel animal model of chronic prostate inflammation.

Aim (2) Determination of the capacity of alternative methods of inciting prostate inflammation to induce chronic inflammation in the context of dietary consumption of PhIP.

Aim (3) Determination of the ability of viral induced prostate inflammation to accelerate prostate carcinogenesis in a rat model of dietary (PhIP) induced prostate cancer.

In order to accomplish these aims, we outlined the following tasks:

Task 1: *Assessment of the ability of candidate dietary prostate cancer chemopreventive compounds (e.g., broccoli tea, soy, vitamin E, lycopene) to alter DNA mutagenesis and chronic prostate inflammation in an animal model:*

<u>**Year 1 Progress Summary:**</u>

We completed the manuscript for the study in which PhIP was given for 4 weeks or 8 weeks that was described in the preliminary data section of the original proposal. This work was published in *Cancer Research* (see manuscript by Nakai et al., listed below).

Task 1 was completed in part in that we performed a study to administer PhIP with and without broccoli tea extract and the non-steroidal anti-inflammatory compound, celecoxib, which is an inhibitor of cyclooxygenase 2 [This study is designated as the 12-week PhIP#1]. The study consisted of 60 Fisher BigBlue rats divided into the following groups: 10 untreated, 10 broccoli, 10 celecoxib, 10 PhIP, 10 PhIP + broccoli, 10 PhIP + celecoxib. Necropsies were performed at 12 weeks on all animals and all prostate lobes and other tissues were harvested for either mutational analysis or histological analysis. A total of 176 paraffin blocks were generated for the histological studies. We found that at 12 weeks of treatment, both chemopreventative agents decreased mutation frequency in the PhIP treated rat prostate (Fig. 1). For celecoxib, the effect was statistically significant. This data was presented as an abstract at the 16th Japanese Society for Molecular and Cellular Urology and the first author (Dr. Nakai) received an award for this presentation.

In addition, we discovered that after 12 weeks of PhIP treatment all animals had developed prostate atrophy that appeared histologically similar to PIA lesions in the human. This is a remarkable finding in that we can conclude that the consumption of

specific dietary agents alone can produce histological changes of prostate atrophy and inflammation in the rat prostate, and, the data support a novel hypothesis suggesting that atrophic lesions in the human prostate may indeed be related to dietary exposures. This result will open up new areas of investigation into this question. For example, it would be highly feasible and reasonable to ask in a pathological-epidemiological study to determine whether the extent of prostate atrophy correlated with dietary intake of PhIP.

Year 2 Progress Summary:

In relation to this task we have begun and generated substantial progress in a new related avenue of research designed to extend our studies of dietary prevention of PhIP induced prostate carcinogenesis from the rat only into the mouse. There are numerous reasons for this including the fact that mice are much more amendable to genetic manipulation which will allow us ultimately to study the molecular pathways that are altered in response to PhIP that leads to PIN and prostate cancer development.

Further, since PhIP induced prostate cancers remain as early lesions that do not metastasize we reasoned that adding PhIP to the Lo-MYC model of early prostate cancer (Cancer Cell. 4:223-238, 2003), which also does not metastasize, might induce more aggressive prostate cancers that do metastasize. This would, therefore, better recapitulate more of the natural history of prostate cancer in humans.

As a first approach and prerequisite to develop this model we have been characterizing the Lo-MYC mice originally developed by Charles Sawyers et al. This work, has been presented as an abstract to the Annual Meeting of the American Urological Association (see appendix) and a manuscript including these results and additional findings not elaborated upon here is currently (started in Year 3) in preparation.
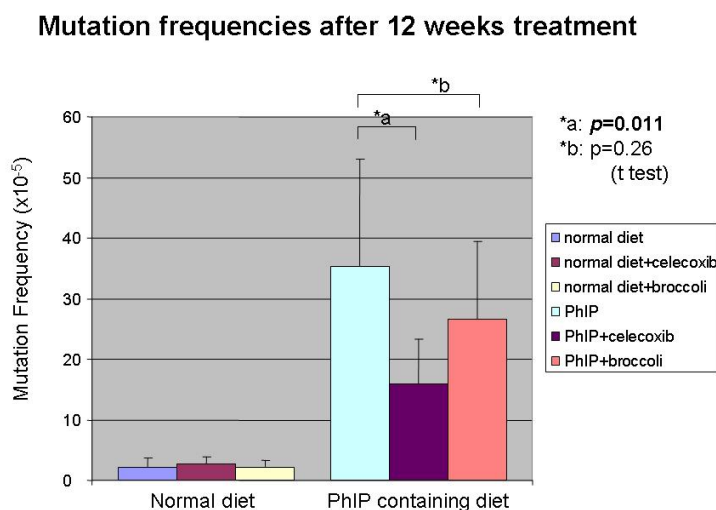

Figure 1 - Reduction in mutations by broccoli tea and celecoxib <mark>week PhIP experiment #1" described below.</mark>

Year 3 Progress Summary:

<mark>*In year #3, the data from Year 1 progress reported above has begun to be further analyzed and is being assembled into a manuscript along with the results of the completed "52-*</mark>

We are now ready to commence the major remaining experiments from Task 1 (Specific Aim 1) and these will be completed using funding outside of the DOD after this award has been terminated. We have obtained the annual renewal to our animal protocol for the studies outlined Task 1. In addition, using resources from this proposal, we have obtained all necessary PhIP and have obtained the formulated diets containing PhIP as well as with the chemopreventive agents.

In summary, a major part of Task 1 has been completed with data still being analyzed. The remainder of Task 1 will be completed in the near future using funding sources outside of the scope of this proposal. **All publications related to these studies will cite appropriate funding from this award by the DOD**.

Task 2: *Determination of the effect of alternative methods of inducing prostate inflammation on the development of chronic inflammation in an established animal model.*

Year 3 Progress Summary:

In efforts to extend these types of experiments into mice as well, we have worked closely with Dr. Charles Bieberich PhD from the University of Maryland, Baltimore County who has successfully induced chronic inflammation in the mouse prostate by intra-urethral injection of *E. coli* into mice. Dr. De Marzo was intimately involved in all aspects of these studies (funded outside of this award) in which he performed all of the pathological analyzes and our laboratory performed a great deal of immunohistochemical staining. This work was submitted as a manuscript that is presently undergoing revision at the *American Journal of Pathology*. The success of this technique in Dr. Bieberich's laboratory has prompted us to consider modifying our approach for the future completion of this Task. Unfortunately at this time, the procedure only generates chronic inflammation when a specific strain of *E. coli* is used in a specific strain of mouse.

As a unique alternative to this, we have begun a new collaboration with Dr. Edward Schaeffer MD PhD, who is a recently hired faculty member in the department of Urology at Johns Hopkins. Dr. Schaeffer has agreed to provide us with a novel source of *E. coli* that has been used successfully in various strains of mice. We will use this bacterium, along with agents originally proposed, to induce inflammation in the rat prostate as outlined in Task 2.

In terms of specific progress towards completing Task 2 in Year 3, we are pleased to report that we have recruited a new post-doctoral fellow, Karen Sfanos PhD, who is currently supported outside of this award by the Department of Urology at Johns Hopkins, to work on this aspect of the study. Thus, the major aspects of all studies in Task 2 will begin in January of 2009 and will continue and be supported by mechanisms

Task 3: *Determination of the ability of viral induced prostate inflammation to accelerate prostate carcinogenesis in a rat model of dietary (PhIP) induced prostate cancer, Months 12-36.*

Year 1 Progress Summary:

In relation to task 3 we completed a study in which 80 rats were treated as follows with 20 rats in each group: 20 rats untreated, 20 rats treated with PhIP, 20 rats treated with PhIP + celecoxib, 20 rats treated with PhIP + broccoli tea. The study was designed such that PhIP was given for 20 weeks in the diet and the celecoxib and broccoli tea was given for the duration of the study, which lasted 52 weeks total [This study is designated 52-Week PhIP#1]. We completed necropsy on all animals and have obtained a total of 619 paraffin blocks for histological analysis. Our preliminary evaluation indicates that there was no major effect by the treatments on the frequency and extent of PIN and intraductal carcinoma in the ventral lobes. However, we are still in the process of quantifying and fully analyzing the results. The results of this study will serve as a baseline for the remaining studies in task 3.

Year 2 Progress Summary:

In a related work we are also interested in developing an understanding of the molecular alterations that occur in PhIP induced prostate neoplastic lesions to determine whether the same somatic genomic alterations that occur in human prostate cancer also occur in this rat model. Toward this end during year 2 we optimized PCR primers to perform bisulfite DNA sequencing of a region of the CpG island of the rat *GSTP1* promoter and have begun to test whether this bisulfite sequencing reaction will be successful after extracting DNA from paraffin blocks from rat prostate tissues. Our plan is to perform laser capture microdissection on the PIN and early carcinoma lesions and compare the results of this to both normal epithelium in treated and untreated rats, as well as to potential changes seen in atrophy that has been induced by PhIP. This information will be used for our prevention aims to monitor molecular changes in PhIP induced cancers as well as morphological changes as originally outlined.

Year 3 Progress:

We have now completed the bulk of the main study for Task 3 and are in the process of analyzing the data. For this we recruited a Urologist-in-Training, Dr. Tsuyoshi Iwata, MD PhD, who is from Osaka University in Japan.

Dr. Iwata was supported by the embedded Urologist-In-Training fellowship as part of this DOD award mechanism. The goal for the traineeship was to provide a first rate experience in molecular pathology of prostate cancer, and we submit that goal has been

achieved. Dr. Iwata's time in our laboratory was highly productive and he was instrumental in performing a number of experiments. Dr. Iwata's current curriculum vita is included in the appendix.

This experiment in Task 3 was designed to determine whether intraprosatic injection of vaccinia virus was adequate to accelerate neoplastic transformation in the rat prostate after 4 weeks of PhIP treatment.

The experimental design was as follows with 5 groups of animals:

PhIP alone (n=10)
PhIP + virus (n=20)
PhIP + vehicle (n=10)
Virus only (n=10)
Control (no injection; n=10)

PhIP treated animals recieved PhIP gavage thrice weekly for 4 weeks, with a prostate injection 2 weeks into PhIP gavage supplementation. 3 months after the completion of PhIP treatment, 3 rats in the PhIP + virus were sacrificed as sentinel animals. The remaining rats were aged for another 3 months and then sacrificed (6 months from PhIP treatment end).

For this experiment we euthanized all animals and dissected prostates from all 60 animals and have obtained paraffin blocks and H&E slides on 138 tissues from these animals.

The preliminary histopathological results suggest that we did not accelerate cancer or PIN using a 4 week PhIP treatment, although we have not yet fully quantified the extent of PIN lesions and this will be done in this subsequent year, again using separate funds. We plan, therefore, as outlined in the orgincal Potential Problems/Alternatives section of Aim 3, to proceed to carry out the experiment in subsequent years in which we will extend the PhIP treatment to 20 weeks.

In carrying out these studies, Dr. Iwata has gained considerable experience in animal handling, animal necropsy and microdissection of the genitourinary organs, PCR, immunohistochemistry, and histopathological interpretation of various prostate lesions.

Other techniques that he has been exposed to are the following: western blotting, quantitative PCR, laser capture microdissection, cell culture, fluorescent in situ hybridization, immunofluorescent staining (single, double and triple labeling) and interpretation, tissue microarray analysis, and computerized image analysis.

Dr. Iwata has been also highly involved with the statistical summarization and analysis of all results, which is being done in conjunction with Dr. Elizabeth Platz , ScD, who is an expert in cancer epidemiology and biostatistics.

An additional very exciting study that is related to the goals of our laboratory in efforts supported by this grant was carried out in mice and is presented in the appendix as an abstract that has been submitted to the upcoming Annual meeting of the AUA. In this study we crossed Lo-MYC mice with mice deficient in *GSTP1* and obtained an accelerated form of prostate cancer induction. Since *GSTP1* is known to be induced by broccoli extracts, we are very excited to continue our studies using broccoli tea to attempt to prevent PhIP-induced prostate cancer in both rats (as originally planned) and in mice.

In relation to the optimization of *GSTP1* promoter methylation assessment in rat tissues that we reported upon in Year 2 progress, we have now obtained excellent results using paraffin embedded sections and are ready to begin to perform laser capture microdissection to determine if *GSTP1* is methylated in PhIP-induced rat PIN and intraductal carcinoma lesions.

KEY RESEARCH ACCOMPLISHMENTS

Year 1

- Completed and published manuscript of some of the key data used for preliminary data in the original grant proposal.

- Completed part of aim 1 by treating PhIP treated Fisher rats with broccoli tea extract and celecoxib.

  - We discovered that both broccoli tea extract and the non-steroidal anti-inflammatory agent celecoxib prevents mutations in all lobes of the prostate in response to PhIP treatment.
  - We discovered that PhIP treatment causes widespread epithelial atrophy in the rat ventral prostate that precedes the development of PIN. This data supports the contention that the PhIP-treated rat model of prostate cancer is a valid model for studying the development of PIA, PIN and early carcinoma.

- Completed a long term study related to task 3 in which we will defined the baseline number and extent of PIN and intraductal cancer lesions in the 52 week model to be used for the remainder of the studies in task 3.

Year 2

- Obtained and extensively characterized, both morphologically and immunohistochemically, the Lo-MYC model of human prostate cancer in order to

ultimately use this model in studies of dietary prevention of PhIP induced cancers.

- Developed a bisulfite DNA sequencing strategy to determine whether *GSTP1* promoter hypermethylation accompanies prostate carcinogenesis in the rat as it does in the human.

- We published a major review article on prostate cancer and inflammation in *Nature Reviews Cancer*, which outlined some of our DOD sponsored research. See Appendix.

Year 3

- A major part of Task 1 has been completed with data still being analyzed.

- We have obtained all necessary PhIP and have obtained the formulated diets containing PhIP as well as with the chemopreventive agents.

- We are now ready to commence the major remaining experiments from Task 1 (Specific Aim 1) and these will be completed using funding outside of the DOD after this award has been terminated.

- We identified a source of a novel strain and source of *E. coli* and we will use this bacterium, along with agents originally proposed, to induce inflammation in the rat prostate as outlined in Task 2.

- We have recruited a new post-doctoral fellow, Karen Sfanos PhD, who is currently supported outside of this award by the Department of Urology at Johns Hopkins, to work on all remaining studies in Task 2 .

- We have now completed the bulk of the main study for Task 3 and are in the process of analyzing the data. For this we recruited a Urologist-in-Training, Dr. Tsuyoshi Iwata, MD PhD, who is from Osaka University in Japan. The preliminary histopathological results suggest that we did not accelerate cancer or PIN using a 4 week PhIP treatment, although we have not yet quantified the extent of PIN lesions and this will be done in this subsequent year.

- We crossed Lo-MYC mice with mice deficient in *GSTP1* and obtained an accelerated form of prostate cancer induction. Since *GSTP1* is known to be induced by broccoli extracts, we are very excited to continue our studies using broccoli tea to attempt to prevent PhIP-induced prostate cancer in both rats (as originally planned) and in mice.

- We further optimized the assay for *GSTP1* promoter methylation assessment in rat tissues that we reported upon in Year 2 progress in that we have now obtained excellent results using paraffin embedded sections and are ready to begin to perform laser capture microdissection to determine if *GSTP1* is methylated in PhIP-

==induced rat PIN and intraductal carcinoma lesions.==

- Other accomplishments of the research team related to this funded project. Our team continues to work and collaborate effectively on studies to examine the etiology of prostate cancer and other prostate diseases.

Our team continues to work and collaborate effectively on studies to examine the etiology of prostate cancer and other prostate diseases. For example working with Dr. Nelson's laboratory we have also obtained preliminary data showing that treatment of neonatal transgenic Lo-MYC mice with the synthetic estrogen, Diethylstibestrol (DES), results in the development of increased levels of inflammation in the mouse ventral prostate as compared to levels induced by DES in wild-type mice or in induced by C-MYC overexpression alone. Thus, we have available another potential model of inducing inflammation in the rodent prostate.

- Shown below are publications related to prostate cancer since the submission of this grant.

  Sutcliffe S, Giovannucci E, **De Marzo AM**, Leitzmann MF, Willett WC, **Platz EA**. Gonorrhea, syphilis, clinical prostatitis, and the risk of prostate cancer. Cancer Epidemiol Biomarkers Prev. 2006 Nov;15(11):2160-6.

  Dunn TA, Chen S, Faith DA, Hicks JL, **Platz EA**, Chen Y, Ewing CM, Sauvageot J, Isaacs WB, **De Marzo AM**, Luo J. A Novel Role of Myosin VI in Human Prostate Cancer. Am J Pathol. 2006 Nov;169(5):1843-54.

  De Marzo AM, **Platz EA**, Epstein JI, Ali T, Billis A, Chan TY, Cheng L, Datta M, Egevad L, Ertoy-Baydar D, Farree X, Fine SW, Iczkowski KA, Ittmann M, Knudsen BS, Loda M, Lopez-Beltran A, Magi-Galluzzi C, Mikuz G, Montironi R, Pikarsky E, Pizov G, Rubin MA, Samaratunga H, Sebo T, Sesterhenn IA, Shah RB, Signoretti S, Simko J, Thomas G, Troncoso P, Tsuzuki TT, van Leenders GJ, Yang XJ, Zhou M, Figg WD, Hoque A, Lucia MS. A working group classification of focal prostate atrophy lesions. Am J Surg Pathol. 2006 Oct;30(10):1281-91.

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  Sutcliffe S, Zenilman JM, Ghanem KG, Jadack RA, Sokoll LJ, Elliott DJ, Nelson WG, **De Marzo AM**, Cole SR, Isaacs WB, **Platz EA**. Sexually transmitted infections and prostatic inflammation/cell damage as measured by serum prostate specific antigen concentration. J Urol. 2006 May;175(5):1937-42.

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De Marzo AM, Nakai Y, Nelson WG. Inflammation, atrophy, and prostate carcinogenesis. Urol Oncol. 25:398-400, 2007.

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Sutcliffe S, Rohrmann S, Giovannucci E, Nelson KE, **De Marzo AM**, Isaacs WB, Nelson WG, Platz EA.   Viral infections and lower urinary tract symptoms in the third national health and nutrition examination survey. J Urol. 178:2181-5, 2007.

Sutcliffe S, Giovannucci E, Gaydos CA, Viscidi RP, Jenkins FJ, Zenilman JM, Jacobson LP, **De Marzo AM**, Willett WC, Platz EA. Plasma antibodies against Chlamydia trachomatis, human papillomavirus, and human herpesvirus type 8 in relation to prostate cancer: a prospective study. Cancer Epidemiol Biomarkers Prev. 16:1573-80, 2007.

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tumor-tolerance systems. J Clin Invest. 2007 Nov;117(11):3383-92.

REPORTABLE OUTCOMES

- Completed manuscripts:

Nakai Y, Nelson WG and **De Marzo AM**. The dietary charred meat carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine acts as both a tumor initiator and promoter in the rat ventral prostate. Cancer Res, Feb 1;67(3): 1378-84, 2007.

De Marzo AM, Platz EA, Sutcliffe S, Xu J, Gronberg H, Drake CG, Nakai Y, Isaacs WB, and Nelson WG. Inflammation in prostate carcinogenesis. Nat Rev Can 7(4):256-269, 2007.

- Abstracts:

Yasutomo Nakai, William G. Nelson, **Angelo M. De Marzo.** The effect of the selective COX-2 inhibitor celecoxib to the early phase of the PhIP induced rat prostate carcinogenesis. Presented at the 16th Japanese Society for Molecular and Cellular Urology, Japan, 2007.

Tsuyoshi Iwata, Charles J. Bieberich, **Angelo M De Marzo**. Paradoxical downregulation of C-MYC and upregulation of NKX3.1 proteins during invasion in the Lo-MYC mouse prostate. Presented to the American Urological Association Annual Meeting, Orlando FL, May 16-22, 2008.

Tsuyoshi Iwata*, Denise Schultz, Matthew Vaughn, Srinivasan Yegnasubramanian, William G Nelson, **Angelo M De Marzo**. Glutathione S-transferase pi (GSTP1) deficiency accelerates prostate carcinogenesis in the Lo-MYC mouse. Submitted to the American Urological Association Annual Meeting, Chicago, IL, April 25-30, 2009.

CONCLUSIONS AND "SO-WHAT"

We discovered that PhIP treatment causes widespread epithelial atrophy in the rat ventral prostate that precedes the development of PIN. This data supports the contention that the PhIP-treated rat model of prostate cancer is a valid model for studying the development of PIA, PIN and early carcinoma. The results raise the novel hypothesis that suggests that the extensive atrophy that is seen in men with prostate cancer, and which has been associated with PIN and prostate cancer, may be caused by ingestion of cooked meats. This result will open up new areas of investigation into this question. For example, it would be highly feasible and reasonable to ask in a pathological-epidemiological study to determine whether the extent of prostate atrophy correlated with dietary intake of PhIP. Also, broccoli and celebrex can decrease

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REFERENCES
- None

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APPENDICES
- Completed manuscripts:

Nakai Y, Nelson WG and **De Marzo AM**. The dietary charred meat carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine acts as both a tumor initiator and promoter in the rat ventral prostate. Cancer Res, Feb 1;67(3): 1378-84, 2007.

De Marzo AM, Platz EA, Sutcliffe S, Xu J, Gronberg H, Drake CG, Nakai Y,   Isaacs WB, and Nelson WG. Inflammation in prostate carcinogenesis. Nat Rev Can 7(4):256-269, 2007.

- Abstracts:

Yasutomo Nakai, William G. Nelson, **Angelo M. De Marzo.** The effect of the selective COX-2 inhibitor celecoxib to the early phase of the PhIP induced rat prostate carcinogenesis. Presented at the 16th Japanese Society for Molecular and Cellular Urology, Japan, 2007.

Tsuyoshi Iwata, Charles J. Bieberich, **Angelo M De Marzo**. Paradoxical downregulation of C-MYC and upregulation of NKX3.1 proteins during invasion in the Lo-MYC mouse prostate. Presented to the American Urological Association Annual Meeting, Orlando FL, May 16-22, 2008.

Tsuyoshi Iwata*, Denise Schultz, Matthew Vaughn, Srinivasan Yegnasubramanian, William G Nelson, **Angelo M De Marzo**. Glutathione S-transferase pi (GSTP1) deficiency accelerates prostate carcinogenesis in the Lo-MYC mouse. Submitted to the American Urological Association Annual Meeting, Chicago, IL, April 25-30, 2009.

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The Dietary Charred Meat Carcinogen 2-Amino-1-Methyl-6-Phenylimidazo[4,5-b]Pyridine Acts as Both a Tumor Initiator and Promoter in the Rat Ventral Prostate

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Abstract

Exposure of Fisher344 rats to 2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine (PhIP), a heterocyclic amine in cooked meat, causes cancer in the rat ventral prostate, while sparing the dorsolateral and anterior lobes. Uncovering the molecular mechanisms of the lobe specificity of PhIP-induced rat prostate cancer may provide clues to the pathogenesis of human prostate cancer, which is also lobe selective. We examined the prostate and other organs for mutation frequencies using transgenic Fisher344 rats (Big Blue rats) after PhIP treatment. After PhIP treatment for as early as 4 weeks, the colon, spleen, seminal vesicles, and all lobes of the prostate had significantly elevated mutation frequencies compared with the saline-treated control group, and the differences became even greater after 8 weeks. $G:C \rightarrow T:A$ transversions were the predominant type of mutation. After 8 weeks of treatment with PhIP, the Ki-67 index was increased ($P < 0.001$) in the ventral prostate, but not in the dorsolateral or anterior prostate. An increase in the number of stromal mast cells and macrophages was seen in the ventral prostate, but not in the other prostatic lobes. The apoptotic index also increased in the ventral lobe only. The increased proliferation and cell death in response to PhIP indicates that in addition to PhIP acting as an "initiator" of cancer, PhIP is also acting like an organ- and lobe-specific tumor "promoter." The prostate lobe-specific infiltration of mast cells and macrophages in response to PhIP suggests a potential new mechanism by which this dietary compound can increase cancer risk—by prompting inflammation. [Cancer Res 2007;67(3):1378–84]

Introduction

Prostate cancer is the most common noncutaneous malignancy and the second leading cause of cancer-related death in men in the United States. Studies of identical twins have revealed a strong hereditary component to prostate cancer, accounting for ∼ 40% of the overall risk (1). Because roughly 60% is unaccounted for by heredity, however, a significant component of the risk for developing prostate cancer must be related to environmental exposures. What are the environmental exposures that increase prostate cancer risk?

There are two long-standing findings that may provide help in answering this question: (a) the incidence and mortality rate of prostate cancer vary worldwide, with the highest rates in the United States and the lowest rates in China and Japan (2); and (b) there is an increased risk of prostate cancer in men who immigrated to the United States from China and Japan that begins within one generation after migration (3–5). One major distinction between men from Western cultures and those from East and Southeast Asian cultures is a marked difference in diet. Traditionally, diets rich in vegetables and fruits are consumed in Southeast Asian countries, whereas diets rich in red meat and animal fat, and low in vegetables and fruits, are consumed in Western cultures. Although not unequivocal, there is epidemiologic evidence of a link between prostate cancer incidence and mortality and consumption of red meat and animal fats (6–9).

The biological mechanism by which a diet rich in red meat leads to cancer has not been fully established. Although red meats contain polyunstaturated fatty acids that may accelerate cancer formation by a number of mechanisms (i.e., free-radical formation during oxidative stress, effects on sex hormone levels, direct signaling affecting cell growth and apoptosis; ref. 10), another mechanism may relate to the cooking of meat at high temperatures (e.g., grilling, broiling or frying), which results in the formation of heterocyclic amines (HCA; ref. 11). HCAs can be metabolized to biologically active metabolites that form DNA adducts, which lead to mutations and to organ-specific cancers. 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is the most abundant HCA present in well-done and charred meats (12). Exposure of laboratory rats to PhIP in the diet results in several tumor types, including carcinomas of the intestine in both sexes, in the mammary gland in females, and in the prostate in males (11, 13–15). Cancers in these tissues in humans have all been associated with PhIP exposure to some extent (6, 16–18), and all of these cancers are common in Western countries and infrequent in Southeast Asian countries (19). These same cancers are increasing rapidly in Japan in parallel with the "westernization" of the diet (20).

Exposure of Fisher344 rats to dietary PhIP for 20 weeks results in the development of localized invasive prostatic carcinomas when examined after ∼ 52 weeks (21, 22). The carcinomas are lobe specific in that they develop in the ventral lobe of the prostate but not the dorsolateral or anterior lobes (21). This finding is intriguing because human prostate cancer is also zone specific, in that most cancers arise in the peripheral zone with less arising in the transition zone and almost none arising in the central zone (23). Therefore, uncovering the molecular mechanisms of the lobe specificity of PhIP-induced cancer in the rat prostate may provide clues to the molecular mechanisms for the lobe specificity of cancer in the human prostate.

There are several possible explanations for the lobe selectivity of PhIP-induced cancers in the rat. Cancer induction requires

both "initiation" and "promotion." Exposures that initiate cancer are known to induce mutations, whereas those that promote cancer are known to increase the proliferation index. It is not clear whether the ventral lobe of the rat prostate is undergoing selective initiation, promotion, or both in response to PhIP.

In terms of initiation, the accumulation of PhIP-DNA adducts in response to dietary exposure has been examined in the prostate lobes separately, and all lobes similarly form these adducts (21). It is clear, however, that whereas adducts are required for mutations, they may not be sufficient (24). The mutation frequency and spectra in response to PhIP treatment can be monitored using Fisher344 "Big Blue rats," which are transgenic for a phage λ shuttle vector (25). Stuart et al. used this model to show that after as little as 61 days of dietary PhIP exposure, there is an ∼20-fold increase in the mutation frequency in the rat prostate (26). Because the entire rat prostate was used in the study by Stuart et al., the lobe specificity of prostate mutations in the rat in response to PhIP has not been previously examined (26). Thus, it is critical to determine whether the ventral lobe is more susceptible to PhIP-induced mutations because this could help to explain the lobe specificity of PhIP-induced prostate cancer.

In terms of tumor promotion, a proliferative index has been assessed in all prostate lobes in response to 4 weeks of PhIP treatment (21). Although Shirai reported a small but significant increase in 5-bromodeoxyuridine uptake in the ventral and dorsolateral lobes in response to PhIP, the lobe specificity of mutations, proliferation rate, and histopathologic changes have not been examined in the same setting in the rat.

Another potential explanation for the lobe selectivity of PhIP-induced prostate cancer may relate to inflammation because several animal models of cancer have been shown to require an inflammatory response for the cancers to occur (27, 28), and the ventral prostate is known to develop spontaneous chronic inflammation in Fisher344 rats that is associated with aging. The mechanisms by which inflammation can increase cancer risk are diverse and may involve initiation, promotion, or both (27). It is important, therefore, to determine whether inflammatory cell infiltrates differ in the different prostate lobes in response to PhIP.

In the present study, we addressed further the molecular mechanisms for the lobe specificity of prostate cancer in the Fisher344 rat by studying the mutation frequency and spectra in each lobe (separately isolated) of the prostate after short-term PhIP treatment. We also compared the mutation frequency and spectra to those found in other organs that are targets of PhIP-induced carcinogenesis (colon and spleen) and those that are not (liver, kidney, and seminal vesicles). In addition, we examined histopathologic and immunophenotypic changes in response to PhIP exposure in each prostate lobe, including cell proliferation rate, and inflammatory cell infiltrates.

Materials and Methods

Chemicals. PhIP hydrochloride was obtained from the NARD Institute (Osaka, Japan) with purity above 99.9%.

Animals and treatment. Male Big Blue transgenic Fisher344 rats (13–15 weeks old) were purchased from Stratagene (La Jolla, CA). Rats were housed in an animal facility maintained on a 12-h light-dark cycle, at a constant temperature (22 ± 2°C) and relative humidity (55 ± 15%). Tap water and food were available *ad libitum*. PhIP was suspended in saline (14 mg/mL) and was given to the rats intragastrically by gavage at the

dose of 70 mg/kg (5 mL/kg) thrice a week for 4 weeks (n = 3) and 8 weeks (n = 3). For the control group, 5 mL/kg of saline was given intragastrically thrice a week for 4 weeks (n = 3) and 8 weeks (n = 3). The animal weights were recorded once per week until the end of the experiment. Rats were euthanized by CO_2 asphyxiation at day 30 for the 4-week treatment group and at day 60 for the 8-week treatment group. All prostate lobes were dissected separately into anterior, dorsolateral complex, and ventral lobes. In addition, the seminal vesicles, liver, kidney, spleen, and colon were immediately dissected. Half of each tissue was flash-frozen in liquid nitrogen and then stored at −80°C. The rest of the tissues were fixed in 10% buffered formalin and examined by routine light microscopy.

Isolation of DNA. DNA was isolated using the RecoverEase protocol (Stratagene). Briefly, 40 to 60 mg of tissue from the different prostate lobes (anterior, ventral, and dorsolateral), seminal vesicles, liver, kidney, spleen, and liver were disaggregated using a Wheaton Dounce tissue grinder, and cell nuclei were collected by centrifuge at 1,100 × g for 12 min. Nuclear pellets were treated with proteinase K and RNAce-It (Stratagene) for 45 min at 50°C and were dialyzed against 10 mmol/L Tris/1 mmol/L EDTA buffer (pH 7.5) for 48 h. The viscous DNA was stored at 4°C until packaging.

Lambda *cII* mutation analysis. Big Blue rats contain a λLIZ shuttle vector that includes the *cII* gene, which is the target for the mutagenesis studies. The *cII* gene plays a critical role in the decision between lysis or lysogeny of λ phage following infection of *Escherichia coli*. In *hfl⁻* bacteria, the cII protein is not degraded, and it activates transcription of the *cI* repressor. The cI protein, in turn, inhibits the transcription of several genes essential for the lytic response. This results in continuous lysogeny and with no plaque formation. When there is an inactivating mutation in the *cII* gene, the *cI* gene is not transcribed, and plaques arise, which enables the number of mutant plaques under selective conditions (24°C) to be determined. Because the λLIZ shuttle vector contains the temperature-sensitive *cI*857 mutation, which inactivates the cI repressor at 37°C, the phage multiplies through the lytic cycle under nonselective conditions (37°C), resulting in plaque formation regardless of the status of the *cII* gene. This allows the calculation of the number of recovered phage genomes or the phage titer. The mutation frequency is calculated by dividing the number of mutant plaques by the phage titer.

We used the λ *Select-cII* mutation detection system for Big Blue rodents (Stratagene) for the lambda *cII* assay. The kit contains the Transpack packaging extract and *hfl⁻ E. coli* G1250. Genomic DNA (8 μL) was incubated in the red tube of the Transpack packaging extract for 90 min at 30°C, and then 12 μL of the Transpack packaging extract from the blue tube was transferred to the red tube and incubated for 90 min at 30°C. Following the incubation, 1.1 mL of SM buffer [100 mmol/L NaCl, 8 mmol/L $MgSO_4 \cdot 7H_2O$, 50 mmol/L Tris-HCl (pH 7.5), 0.01% gelatin] was added to each packaging reaction. One hundred microliters of packaged λ phage solution was mixed with 200 μL of G1250 at room temperature for 30 min, mixed with 2.5 mL TB1 top agar, and plated on ten 10-cm dishes containing 25 mL bottom agar. The plates were incubated at 24°C for 48 h. For titration, a 100-μL aliquot of a 1:100 dilution of the packaged λ phage solution was mixed with 200 μL of G1250 and 2.5 mL TB1 top agar, plated on three dishes, and incubated at 37°C for 24 h.

Sequencing of *cII* mutants. To validate whether presumptive mutant plaques actually represented phage genomes containing a mutant *cII* gene, plaques obtained at 24°C were analyzed for *cII* gene sequence mutations. When the number of mutant plaques exceeded 40 per assay, 10% to 30% of mutant plaques were sequenced for the *cII* gene. The *cII* gene was amplified by PCR using primer pair 5′-CCGCTCTTACACATTCCAGC-3′ and 5′-CCTCTGCCGAAGTTGAGTAT-3′. The amplified *cII* gene length is 294 bp, and the total PCR fragment is 476 bp. The amplified *cII* gene was purified by the Rapid 96 PCR Purification System (Marligen Bioscience, Ijamsville, MD). DNA sequencing was done using an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA), and the sequence was analyzed using Sequencher v4.1.4 (Gene Codes Corporation, Ann Arbor, MI).

Antibodies. Anti–Ki-67 antibody (rabbit polyclonal, dilution 1:4,000) was from Novocastra (Newcastle-upon-Tyne, United Kingdom). Antirat CD68

antibody (mouse monoclonal, clone ED1, dilution 1:2,000) was from Serotec (Oxford, United Kingdom). Anti-glutathione-*S*-transferase antibody (rabbit polyclonal, dilution 1:1,250) was from Lab Vision (Fremont, CA). Anticleaved caspase 3 antibody (rabbit polyclonal) was from Cell Signaling Technology (Beverly, MA).

Histology and immunohistochemistry. Histologic examination was done on paraffin-embedded sections after H&E staining. Immunohisto-chemistry was done using the EnVision+ detection system (DAKO, Carpinetria, CA). Briefly, paraffin sections were deparaffinized and rehydrated through a graded alcohol series. For Ki-67 and GSTP1 staining, steam heating in Target Retrieval Solution (DAKO) for 40 min was done. For cleaved caspase 3 and CD68 staining, steam heating in Antigen Unmasking Solution (Vector Laboratories, Burligame CA) for 20 min was done. Primary antibodies were incubated for 45 min at room temperature (Ki-67, CD68, cleaved caspase 3) or overnight at 4°C (GSTP1). Slides were counterstained with hematoxylin.

Assessment of immunostaining. Ki-67 and caspase 3 staining was evaluated by quantitative analysis using the Chromavision ACIS (Clarient, San Juan Capistrano, CA). At least 10 regions were randomly chosen from each rat, and prostatic epithelial cells were circled on the computer monitor, and brown area (positive staining) and blue area (nuclei not immunostained) in the regions were counted. The Ki-67 labeling index and apoptosis index were obtained by dividing the brown area by the brown area plus the blue area.

Mast cell staining. Paraffin-embedded sections were deparaffinized with xylene and stained with 0.1% toluidine blue in 1% sodium chloride solution. Staining with toluidine blue permits the identification of mast cells because mast cell granules stain metachromatically, resulting in deep purplish-blue granular cytoplasmic staining.

Mast cell and macrophage counting. The number of mast cells and macrophages were counted at a magnification of 100× and 200×, respectively, under light microscopy. At least five regions were selected at random for counting, and the average number in each group was determined.

Statistics. The difference of the number of Ki-67–positive cells, caspase 3–positive cells, mast cell number, and macrophage number was analyzed by two-sided unpaired Student's *t* test and considered statistically different when *P* value was <0.05. The correlation between mutation frequencies and mast cell number and the correlation between mast cell number and Ki-67 index were analyzed by linear regression.

Results

Mutation frequencies. The mutation frequencies of the saline-treated rats (negative controls) represent the spontaneous frequencies in each tissue. The spontaneous mutation frequencies in the control rats were not different between the tissues analyzed both after 4 weeks ($F = 1.86$; $P = 0.14$) or after 8 weeks ($F = 1.36$; $P = 0.30$; one-way factorial ANOVA; Fig. 1). By contrast, after PhIP exposure for either 4 or 8 weeks, the mutation frequencies of the colon, spleen, seminal vesicles, and each lobe of the prostate were significantly greater than their corresponding tissues in the control group (Fig. 1). The liver and kidneys, which do not develop cancer in response to PhIP (11), did not show an increase in their mutation frequencies after exposure to PhIP (Fig. 1).

Mutation spectra. The mutation spectra of the target tissues after 4 and 8 weeks of PhIP treatment are shown in Fig. 2. G:C → A:T transitions, G:C → T:A, G:C → C:G transversions, and single base-pair deletions were significantly increased.

The most frequently deleted base was guanine (data not shown). Overall, these types of mutations are similar to that expected after PhIP treatment (29–31).

Histology and histochemistry. By standard H&E staining, there were no differences in morphology within the epithelial compartment between the various prostate lobes of the PhIP-treated and untreated animals after either 4 or 8 weeks of exposure to PhIP. However, when analyzed for epithelial cell proliferation in response to PhIP treatment by immunostaining for Ki-67, the dorsolateral and anterior lobes showed virtually no change, yet the ventral prostate showed a marked increase in staining (Fig. 3*A–F*). This increase in the proliferative fraction of epithelial cells was statistically significant both after 4 and 8 weeks of PhIP exposure (Fig. 3*G*; 4-week data is not shown). In terms of cell death, very few apoptotic cells were seen in the ventral prostate of the saline-treated rats after 8 weeks, but when treated with PhIP, there was an increase in apoptosis (from 0% to ∼0.1%) as detected by immunostaining for activated caspase 3 (data not shown).
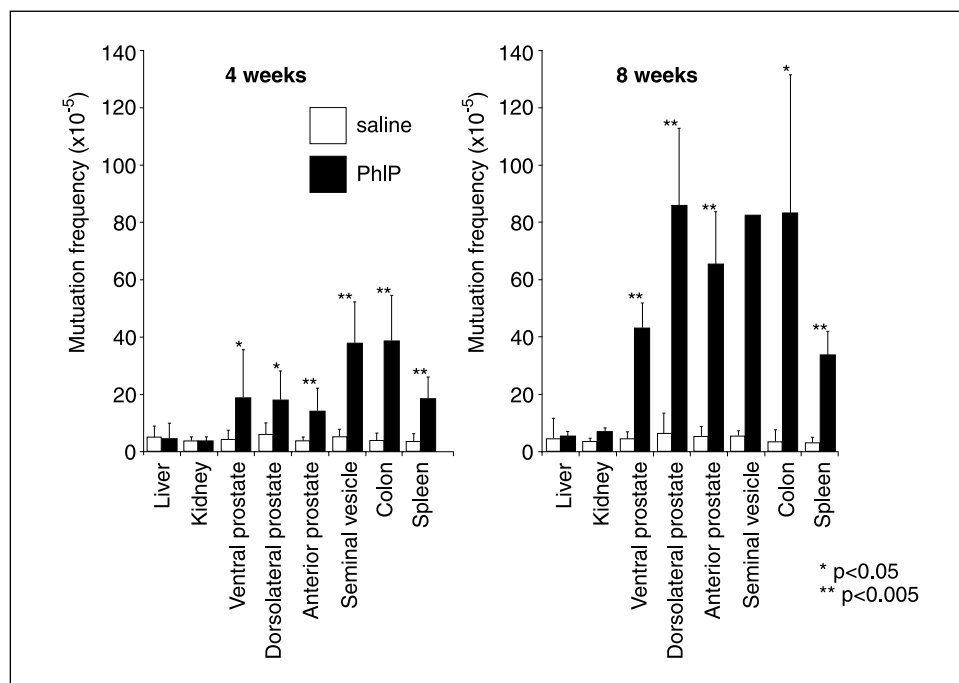

Figure 1. Mutation frequencies after 4 wks (*left*) and 8 wks (*right*) of PhIP treatment. Each tissue and prostate lobe was collected and subjected to λ *cII* mutation analysis. *Columns,* means (*n* = 3); *bars,* SE. Student's *t* test analysis: *, *P* < 0.05; **, *P* ≤ 0.005 compared with the saline group and PhIP group in each tissue.

Figure 2. Mutation frequency and spectra in the *cII* gene after 4 wks (*left*) and 8 wks (*right*) of PhIP treatment. In the control animals, G:C to A:T transition mutation was the most frequently seen mutation. After PhIP treatment, G:C to T:A transversions, followed by −1-bp deletions (most of them were guanine deletion) were the most frequently seen types of mutation.
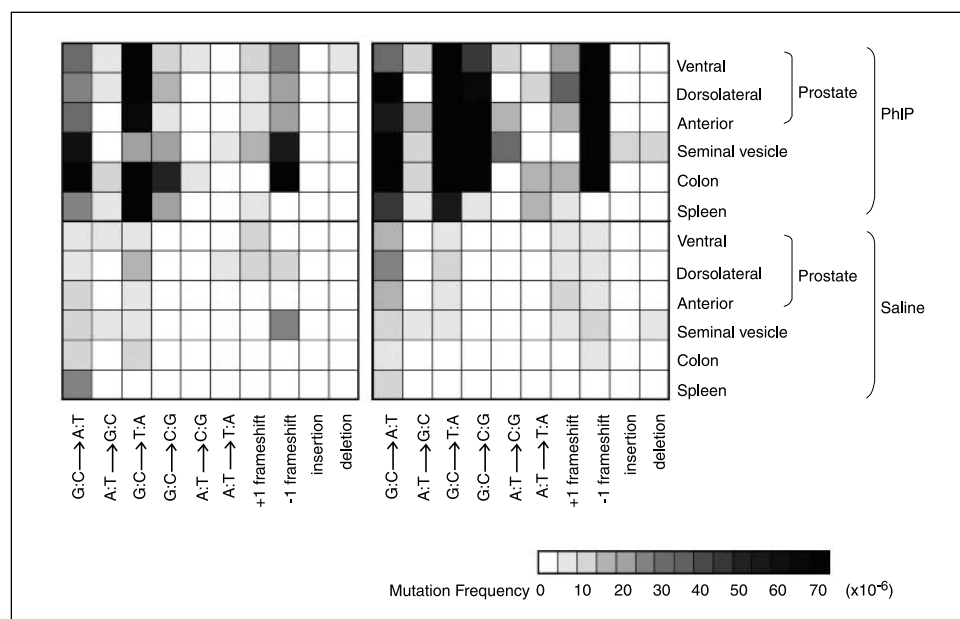
In the stromal compartment of the ventral prostate, standard H&E staining revealed an increase in mononuclear cells in which blue granules were seen in the cytoplasm. These cells were confirmed to be mast cells by staining with toluidine blue (Fig. 4*A* and *B*). After both 4 and 8 weeks of PhIP treatment, the number of mast cells was significantly increased only in the ventral prostate (Fig. 4*E*), and this increase correlated with the mutation frequencies ($r = 0.867$; $P = 0.0003$). Although there was no spatial relation between individual mast cells and proliferating epithelial cells, as determined by Ki-67 staining with toluidine blue counterstaining (data not shown), there was a strong correlation between the number of mast cells and the proliferation index ($r = 0.937$; $P < 0.0001$). The number of macrophages, which were labeled by CD68 staining, was also increased in the ventral prostate only both after 4 and 8 weeks of treatment with PhIP (Fig. 4*C* and *D*). The number of macrophages also correlated with the proliferation index ($r = 0.629$; $P = 0.029$).

Because the π class glutathione *S*-transferase gene (*GSTP1*) is inactivated in human prostate cancer by the promoter region CpG island hypermethylation and can prevent PhIP-induced DNA adduct formation in human prostate cancer cells in culture (32, 33), we also examined the pattern of GSPT1 protein expression in the

Figure 3. Ki-67 staining (*A–F*) of the prostate after 8 wks of saline (*left*) and PhIP (*right*) treatment. *G,* the ratio of the positive cells was analyzed by the Chromavision ACIS II (Clarient, San Juan Capistrano, CA). *Columns,* means of the positive cell ratio (%) of the 30 areas from each group; *bars,* SE. Student's *t* test analysis: *, $P < 0.0001$.
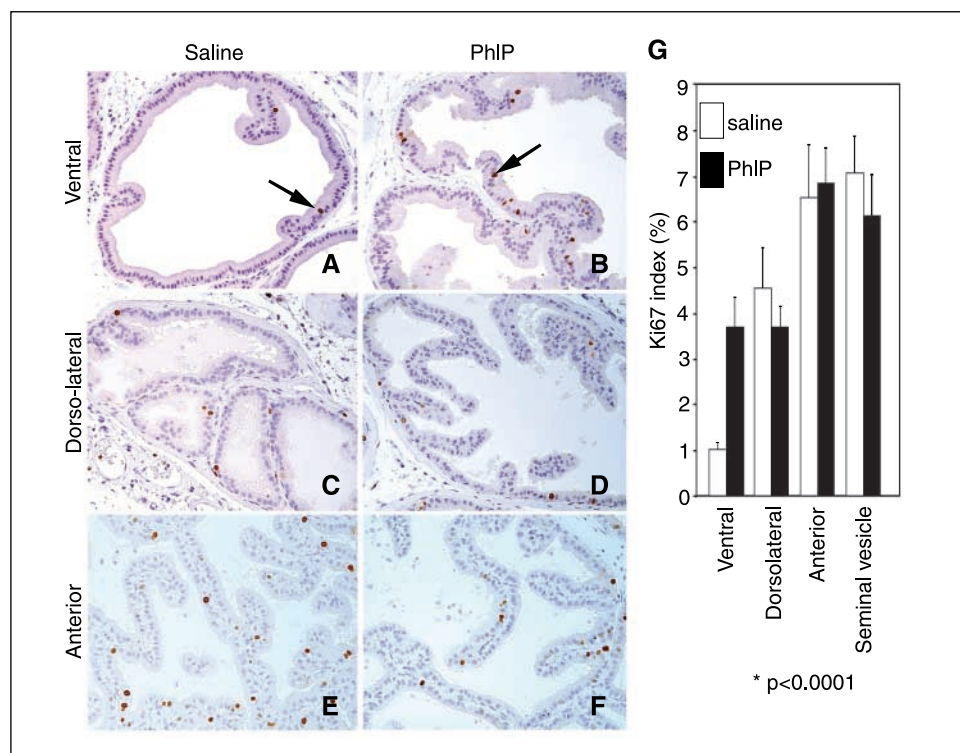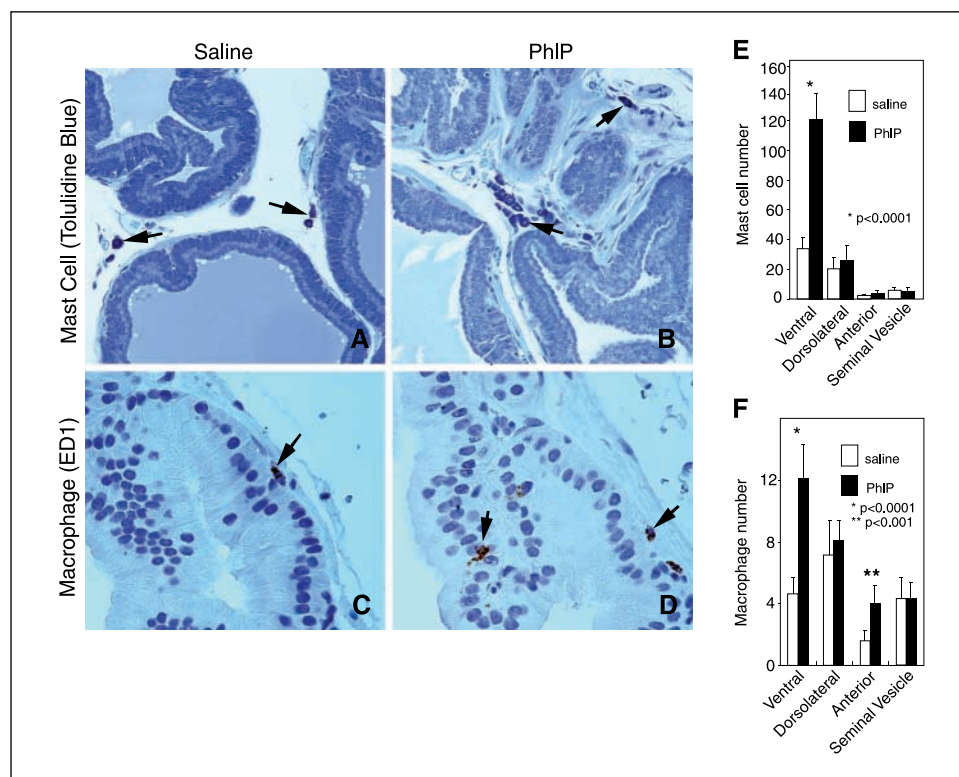
Figure 4. Mast cell (toluidine blue) staining (*A* and *B*) and macrophage (CD68) staining (*C* and *D*) of the ventral prostate. *A* and *C,* saline-treated and (*B* and *D*) PhIP-treated rats (data shown is from the 8-wk treatment groups). *E,* mast cell numbers were counted under microscope at ×100. *F,* macrophage numbers were counted under the microscope at ×200. *Columns,* means of mast cell or macrophage number of the field analyzed in each group; *bars,* SE. *P* values are from the Student's *t* test analysis. *A* and *B, arrows,* mast cells; *C* and *D, arrows,* macrophages.

various lobes of the rat prostate in response to PhIP. Strong GSPT1 staining was seen in basal cells in all lobes, and variable and weaker staining was noted in the luminal cells. However, no change in the extent or pattern of GSTP1 staining was noted after PhIP treatment at either time point.

Discussion

HCAs, of which there are at least 10 chemical forms, are produced from amino acids, creatine/creatinine, and polysaccharide precursors during the high-temperature cooking of meats and fish (12). PhIP, which is the most abundant of the HCAs found in cooked meats, has been shown to be carcinogenic in the prostate, mammary gland, intestine, and lymphoid tissue in experimental animal studies (34–36). The major pathway of HCA activation involves phase I hepatic cytochrome P450–mediated N-hydroxylation followed by phase II esterification of the *N*-hydroxylamines to reactive ester derivatives. These derivatives spontaneously degrade to arylnitrenium ions and form DNA adducts by covalent binding, mainly to the C8 position of guanine. If not repaired, these DNA adducts can result in base substitution mutations, small deletions, and small insertions (37). Cui et al. showed that in human prostate xenografts, PhIP-DNA adducts can be detected after exposing mice to PhIP intragastrically (38), and Nelson et al. showed that in human explants of prostate tissues, *N*-hydroxy PhIP can be metabolized to adduct-forming derivatives (32). These studies indicate that the human prostate can be a target of dietary PhIP-induced adducts.

In this study, we found that the colon, spleen, seminal vesicles, and all lobes of prostate are target tissues of PhIP-induced mutations. In these tissues, 4 weeks was sufficient to induce mutations. The type of mutations most frequently observed were G:C to A:T transitions followed by G:C to T:A transversions, G:C to C:G transversions, and −1-bp deletions in these target tissues. These types of mutations

were similar to that expected after PhIP treatment (29), suggesting that these mutations were caused by PhIP-DNA adducts.

The tissue specificity of PhIP-induced mutations in this study (liver and kidney did not show increases, and the prostate, seminal vesicles, and colon did show increases) suggests that PhIP-induced mutations require cell proliferation—liver and kidney epithelial cells turn over extremely slowly, whereas splenocytes, colonic epithelial cells, prostate epithelial cells, and seminal vesicle epithelial cells have a variable yet much higher turnover rate than the liver or kidney. However, not all of these target tissues of PhIP-induced mutations are the target tissues of PhIP-induced cancer (11). Despite the present study's finding that all prostate lobes and seminal vesicles were target tissues for mutation, only the ventral lobe is known to develop prostate cancer in this rat model. Nagao et al. reviewed the association between DNA adducts, mutation, and cancer incidence and did not find a strong correlation between mutation frequency and cancer incidence (24). This fact suggests that whereas mutations, which are related to the initiation of cancer, are required for malignant transformation, there must be other factors, such as tumor "promoters," required for the cancer occurrence in response to PhIP.

To further examine the link between PhIP-induced mutations and prostate lobe–specific carcinogenesis, we analyzed the prostate lobe–specific phenotypic responses to PhIP. In the dorsolateral prostate, anterior prostate, and seminal vesicle, there was no change in proliferation in response to PhIP. However, in the ventral prostate, there was a significant increase in proliferation after exposure to PhIP. An increase in proliferation in response to PhIP has also been reported in other target tissues. In a PhIP-induced rat mammary cancer model, proliferation of the epithelial cells of the mammary gland terminal end buds, putative sites of origin of carcinomas, increased after exposure to PhIP (39), and Shirai has previously reported an increase in proliferation in all

prostate lobes in response to PhIP (21). It is unclear why we found in the present study an increase in proliferation only in the ventral lobe. Nevertheless, these findings suggest that PhIP is acting as both an initiator and promoter of cancer in the ventral prostate. What might be responsible for this increased proliferation?

One potential mechanism may relate to the finding that PhIP has an estrogenic effect and can stimulate cell proliferation through estrogen receptor α (31, 40). Fujimoto et al. showed that estrogen receptor α mRNA is expressed in the rat ventral prostate (41), and that estrogen and testosterone together up-regulated the expression of estrogen receptor α mRNA (41). In addition, estrogen, along with testosterone, resulted in increased expression of androgen-responsive genes (41). Estrogens can induce prostate inflammation in the rat and have been postulated to play a role in increasing the risk for human prostate cancer (42). Whether estrogen receptor signaling is involved in PhIP-induced prostate carcinogenesis awaits further study.

Another potential novel mechanism by which PhIP may indirectly influence prostate carcinogenesis in the ventral prostate may relate to our finding that mast cells and macrophages accumulated selectively in the ventral prostate in response to PhIP treatment. Recent experiments indicate that mast cell infiltration can enhance carcinogenesis (43, 44). Mast cells contribute to the development of skin cancer in K14-HPV16 transgenic mouse by releasing proteases, such as tryptase and chymase, and stimulating angiogenesis (45). In a 1,2-dimethylhydrazine–induced intestinal tumor model, the incidence of intestinal cancer was significantly reduced in mast cell–deficient Kit^W/Kit^W-v mice, whereas when the mast cells were rescued by bone marrow transplantation, the cancer incidence was the same as the normal mice treated with 1,2-dimethylhydrazine (46). Although the mechanisms by which mast cells contribute to carcinogenesis are not understood, mast cells play an important role in the initiation of inflammation. Mast cells are the only tissue-resident cells with granules containing preformed tumor necrosis factor α, and releasing this cytokine from mast cells is important for the initiation of an inflammatory response (47). In this study, along with mast cell infiltration in the ventral prostate, the number of macrophages was increased in the ventral prostate after PhIP treatment. This increase of macrophages might partly be due to the cytokines released from infiltrating mast cells. Macrophages have been shown clearly to aid in both the initiation and progression of experimental cancers (48–50). Although our study was conducted

for 8 weeks, we have now done additional longer term studies in the same model system. In our preliminary analysis, we have reproduced the findings of Shirai et al. in that the addition of PhIP at 400 ppm to the diet for 20 weeks results in the appearance of intraductal carcinoma lesions at 52 weeks. In this model/paradigm, we have now found similar increases in mast cells, even at 52 weeks, in the PhIP-treated animals. Additional work is needed to determine whether mast cells and/or macrophages are contributing to prostate carcinogenesis in response to PhIP.

In summary, in attempts to begin to elucidate the lobe-specific carcinogenetic effects of the dietary carcinogen PhIP on the rat prostate, we have shown that all prostate lobes accumulate mutations in response to short-term PhIP treatment. Thus, mutations alone, while necessary for cancer formation, do not correlate with subsequent cancer formation. We report for the first time that the ventral prostate selectively responded to PhIP treatment by an increase in stromal mast cells, macrophages, and epithelial cell proliferation. These results suggest that the inflammatory response may help explain the tissue-specific and prostate lobe–specific carcinogenesis in the rat prostate induced by a dietary carcinogen.

Note Added in Proof

After the submission of this manuscript, Borowsky et al. (Neoplasia 2006;8:708–15) reported that PhIP induces acute and chronic inflammation in the prostate prior to inducing PIN and intraductal cancers. Although masts cells and macrophages were not evaluated, and the lobe specificity was not commented upon, these results are consistent with the concept that PhIP may act in part by inducing inflammation in the prostate.

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Inflammation in prostate carcinogenesis

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Abstract | About 20% of all human cancers are caused by chronic infection or chronic inflammatory states. Recently, a new hypothesis has been proposed for prostate carcinogenesis. It proposes that exposure to environmental factors such as infectious agents and dietary carcinogens, and hormonal imbalances lead to injury of the prostate and to the development of chronic inflammation and regenerative 'risk factor' lesions, referred to as proliferative inflammatory atrophy (PIA). By developing new experimental animal models coupled with classical epidemiological studies, genetic epidemiological studies and molecular pathological approaches, we should be able to determine whether prostate cancer is driven by inflammation, and if so, to develop new strategies to prevent the disease.

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Prostate cancer is the most common non-cutaneous malignant neoplasm in men in Western countries, responsible for the deaths of approximately 30,000 men per year in the United States[1]. The number of afflicted men is increasing rapidly as the population of males over the age of 50 grows worldwide. Therefore, finding strategies for the prevention of prostate cancer is a crucial medical challenge. As men in South East Asian countries have a low incidence of prostate cancer that increases rapidly after immigration to the West, this disease is not an intrinsic feature of ageing. The pathogenesis of prostate cancer reflects both hereditary and environmental components. What are the environmental factors and genetic variations that have produced such an epidemic of prostate cancer? Approximately 20% of all human cancers in adults result from chronic inflammatory states and/or chronic inflammation[2–4] (BOX 1), which are triggered by infectious agents or exposure to other environmental factors, or by a combination thereof. There is also emerging evidence that inflammation is crucial for the aetiology of prostate cancer. This evidence stems from epidemiological, histopathological and molecular pathological studies. The objective of this Review is to take a multidisciplinary approach to present and analyse such studies. Because several reviews related to these topics have been published[5–7], here we will focus on new findings and ideas with the purpose of sparking innovative areas of investigation that might ultimately lead to the prevention of prostate cancer.

Enigmas in the aetiology of prostate cancer

As in other cancers, prostate cancer develops through the accumulation of somatic genetic and epigenetic changes, resulting in the inactivation of tumour-suppressor genes and caretaker genes, and the activation of oncogenes[8,9] (TABLE 1). There is also evidence for an underlying genetic instability that might facilitate tumour progression[10,11]. Although these genetic and epigenetic changes are crucial for our understanding of 'how' prostate cancer arises, another key remaining question is 'why' prostate cancer is so common. The most consistent risk factors for the development of prostate cancer are advancing age, family history and race — diet is thought to be an emerging risk factor. To answer the question of why prostate cancer is so prevalent, several puzzling facts regarding its occurrence must be explained. The first enigma is the striking organ selectivity of prostate cancer within the genitourinary system: whereas there are approximately 280,000 new cases of prostate cancer in the US each year, there have been less than 50 reported cases of primary seminal vesicle carcinoma in the English literature[12]. The second unexplained issue is the geographic variation in the incidence of prostate cancer: as compared with the US and Western Europe, the incidence and mortality rates for prostate cancer are much lower in Southeast and East Asia[13]. Chinese and Japanese men who immigrate to the west acquire higher prostate cancer risks within one generation[14], supporting an effect of the environment

At a glance

- Prostate cancer is the most common form of non-skin cancer in men in developed countries. The cause(s) of prostate cancer have not yet been clarified. Although heritable factors are implicated, immigration studies indicate that environmental exposures are also important.

- Chronic infection and inflammation cause cancer in several organs including the stomach, liver and large intestine. Data from histopathological, molecular histopathological, epidemiological and genetic epidemiological studies show that chronic inflammation might also be important in prostate carcinogenesis.

- The source of intraprostatic inflammation is often unknown, but might be caused by infection (for example, with sexually transmitted agents), cell injury (owing to exposure to chemical and physical trauma from urine reflux and prostatic calculi formation), hormonal variations and/or exposures, or dietary factors such as charred meats. The resultant epithelial cellular injury might cause a loss of tolerance to normal prostatic antigens, resulting in a self-perpetuating autoimmune reaction.

- Exposures to infectious agents and dietary carcinogens are postulated to directly injure the prostate epithelium, resulting in the histological lesions known as proliferative inflammatory atrophy (PIA), or proliferative atrophy. These lesions are postulated to be a manifestation of the 'field effect' caused by environmental exposures.

- Despite a strong genetic component to prostate cancer risk, no highly penetrant hereditary prostate cancer genes have been uncovered to date. Although complex, genetic variation in inflammatory genes is associated with prostate cancer risk.

- Several challenges remain regarding the inflammation hypothesis in prostate cancer, including the determination of the cause(s) of chronic inflammation in the prostate, an understanding of the cellular and molecular biology of the immune response in the prostate, whether inflammatory cells are truly causative in the process, and the determination of the target cell types within the proposed precursor lesions of prostate cancer.

- The refinement and application of new epidemiological approaches, including high-throughput genetic epidemiology, improved rodent models of prostate inflammation and cancer, and advances in the application of molecular techniques to histopathological studies should provide insights into the cause of prostate inflammation and its relevance to prostate carcinogenesis.

Prostatic intraepithelial neoplasia
A lesion characterized by cells with neoplastic features, which line pre-existing acini and ducts. PIN represents the most likely precursor to many prostate cancers.

Benign prostatic hyperplasia
Non-cancerous enlargement consisting of excess glands and stroma affecting the transition zone of the prostate.

Urine reflux
During urination, urine flows from the bladder through the prostatic urethra and into the penile urethra. Urine reflux occurs when urine flows inadvertently into the prostatic ducts, permeating large portions of the prostatic acini.

on prostate cancer development. A third key unsolved problem is the zonal predilection of prostate cancer. Most cancer lesions occur in the peripheral zone of the gland, fewer occur in the transition zone, and almost none arise in the central zone[15] (FIG. 1).

Inflammation and prostate cancer: the role of PIA

Histologically, most lesions that contain either acute or chronic inflammatory infiltrates in the prostate are associated with atrophic epithelium or focal epithelial atrophy[16–18]. Perhaps correspondingly, focal areas of epithelial atrophy are common in the ageing prostate[16,19], and often encompass a large fraction of the peripheral zone, where atrophy most often occurs[19,20]. Compared with normal epithelium, there is an increased fraction of epithelial cells that proliferate in focal atrophy lesions[18,21,22], and we have proposed the term proliferative inflammatory atrophy (PIA) for most of these atrophic lesions[18,23]. Not all focal prostate atrophy lesions show increased inflammatory cells, and for these the term proliferative atrophy might be used. In morphological studies we and others have observed transitions between atrophic epithelium and adenocarcinoma[16,24,25], and frequent transitions between areas of PIA and/or proliferative atrophy with high grade prostatic intraepithelial neoplasia (PIN)[18,26]. Although there is evidence for somatic genetic changes

in PIA and proliferative atrophy, it seems from the studies published so far that most PIA and proliferative atrophy lesions do not harbour clonal genetic alterations (BOX 2). Tissue samples from patients with benign prostate hyperplasia (BPH), which occurs in the transition zone of the prostate (FIG. 1), have areas with markedly increased numbers of chronic inflammatory cells. In these areas, in almost all cases, the epithelium seems to be atrophic, indicating that these regions can be considered PIA of the transition zone.

Several key molecular pathways involved in prostate cancer have also been shown to be altered in PIA lesions (BOX 3). For example, the protein products of three prostate tumour-suppressor genes: *NKX3.1* (REF. 27), *CDKN1B*, which encodes p27 (REFS 18,23), and phosphatase and tensin homologue (*PTEN*) (A.M.D. and D. Faith, unpublished observations) are all downregulated in focal atrophy lesions. These genes are highly expressed in normal prostate epithelium, and frequently decreased or absent in PIN and prostate cancer. In addition, one allele of their corresponding genetic loci is frequently deleted in carcinomas (TABLE 1), and forced overexpression of each of these genes causes decreased growth of prostate cancer cells in culture. Finally, animal models with targeted disruption of either one or two alleles of the corresponding mouse genes develop prostate hyperplasia, PIN and/or invasive carcinoma[28].

What is the source of prostatic inflammation?

In most cases, the cause of prostatic inflammation is unclear. Various potential sources exist for the initial inciting event, including direct infection, urine reflux inducing chemical and physical trauma, dietary factors, oestrogens, or a combination of two or more of these factors (FIG. 2). Furthermore, any of these could lead to a break in immune tolerance and the development of an autoimmune reaction to the prostate.

Infectious agents. Many different pathogenic organisms have been observed to infect and induce an inflammatory response in the prostate. These include sexually transmitted organisms, such as *Neisseria gonorrhoeae*[29], *Chlamydia trachomatis*[30], *Trichomonas vaginalis*[31] and *Treponema pallidum*[32], and non-sexually transmitted bacteria such as *Propionibacterium acnes*[33] and those known to cause acute and chronic bacterial prostatitis, primarily Gram-negative organisms such as *Escherichia coli*[34]. Although each of these pathogens has been identified in the prostate, the extent to which they typically infect this organ varies. For example, *T. pallidum* is a very rare cause of granulomatous prostatitis[32], which is itself a rare pattern of prostate inflammation. In the pre-antibiotic era before 1937, a large proportion of other sexually transmitted infections (STIs, predominantly gonorrhea) resulted in severe prostatic inflammation or prostatic abscess[29]. However, since the introduction of antibiotics this proportion has decreased dramatically, presumably owing to treatment before progression to the prostate. Despite this decline, asymptomatic infection and inflammation of the prostate can still occur. In their study of gonorrhea,

Box 1 | **Molecular mechanisms of inflammation-induced cancers**

Chronic inflammation is implicated in the development of a diverse range of human cancers, with overwhelming evidence causally linking it to cancer of the liver, stomach, large intestine, biliary tree and urinary bladder, and significant evidence to link it to cancer of the oesophagus, lung and pancreas. Many of these cancers are associated with infectious agents and/or a defined environmental exposure(s). Inflammation often collaborates with environmental exposures, such as dietary derived toxins, to increase cancer risk even further[132]. The molecular mechanisms that underlie the pathogenesis of inflammation-associated cancer are complex, and involve both the innate and adaptive immune systems[3,133,134,135]. Although viral oncogenes can contribute directly to neoplastic transformation, neither infection nor pathogen-encoded oncogenes are required for inflammatory cells to induce cancer[136]. Indeed, highly reactive chemical compounds, including superoxide, hydrogen peroxide, singlet oxygen and nitric oxide are released from activated phagocytic inflammatory cells of the innate immune system, and can cause oxidative or nitrosative damage to DNA in the epithelial cells, or react with other cellular components such as phospholipids, initiating a free-radical chain reaction[2]. The result is that many host epithelial cells are damaged and killed, and in order to preserve the barrier function of epithelia, these cells must be replaced by cell division from resident progenitor and/or stem cells. Epithelial cells that undergo DNA synthesis in the setting of these DNA-damaging agents are at an increased risk of mutation. That oxidant or nitrosative stress is important for driving prostate cancer formation is bolstered by epidemiological data, which indicate that the consumption of certain types of dietary antioxidants is associated with reduced prostate cancer risk[137]. Inflammatory cells also secrete cytokines that promote epithelial cell proliferation and stimulate angiogenesis[138]. In terms of disease progression, inflammatory cells migrate readily through the extracellular matrix as a result of the release of proteolytic enzymes and their inherent motile nature. Therefore, they might facilitate epithelial cell invasion into the stromal and vasculature compartments and, ultimately, the metastasis of tumour cells[133,134]. In another mechanism, the disruption of cytokine production and regulation, including cytokine deficiencies, lead to increased inflammation and cancer, whether in response to infection with a commensal organism or to chemical carcinogens[139]. A final mechanism is that certain immune responses can directly dampen cell-mediated anti-tumour immune surveillance mechanisms, thereby averting an immune reaction against the tumour that could potentially eliminate the cancer[90].

Handsfield and colleagues[35] cultured *N. gonorrhoeae* in expressed prostate fluid after urination in 93% of men with asymptomatic gonorrhea.

Viruses can also infect the prostate, and human papillomavirus (HPV), human herpes simplex virus type 2 (HSV2), cytomegalovirus (CMV) and human herpes virus type 8 (HHV8) have been detected in the prostate[36–38]. How frequently these agents infect the prostate, and whether they elicit an inflammatory response, is largely unknown. In conclusion, many different pathogens can infect the prostate. Whereas some of these are associated with inflammation, others have not been detected in association with inflammation. Because many additional bacterial sequences[39], and now a new viral sequence[40], can be found in prostate tissue in the absence of an ability to culture any of these organisms using traditional means, it is still possible that in analogy to *H. pylori* gastritis, researchers have missed a previously unidentified pathogen associated with most inflammatory lesions in the prostate.

Several epidemiological studies of STIs and prostate cancer have been undertaken (BOX 4). Adding weight to the argument for a link between inflammation and prostate cancer are data indicating that users of anti-inflammatory agents have a reduced risk of prostate cancer. Prospective and case–control studies, including a relatively small prospective analysis that we conducted, suggest a reduction of ~15–20% in the risk of prostate cancer in regular users of aspirin or non-steroidal anti-inflammatory drugs (NSAIDs) compared with non-users[41,42,43]; however, in a large study by Jacobs *et al.* the effect was seen only in long-term users[44].

Although most studies investigating clinical prostatitis in relation to prostate cancer reported a positive association[45,46], many of these studies might have been susceptible to detection bias. In our work, there was no association between clinical prostatitis and prostate cancer among men with an equal opportunity for prostate cancer screening by serum prostate specific antigen (PSA) testing, except in men diagnosed with cancer at a young age[47]. Although it is unclear why the effect was seen only in early-onset prostate cancer, it is possible that clinical prostatitis is associated with only a subset of prostate cancers that manifest at a relatively young age.

To determine whether inflammation is related to prostate cancer independent of clinical symptoms, it will be crucial to compare the patterns and extent of inflammation in prostate biopsy samples from men with and without carcinoma. As inflammation is so common in prostate specimens, these measurements will need to be quantitative, and will require large sample sizes. There is a US National Institutes of Health (NIH) consensus grading system[48] for histological prostate inflammation, and we will be using this system in a nested case–control study to determine whether asymptomatic prostatic inflammation is associated with prostate cancer using needle biopsy specimens from the Prostate Cancer Prevention Trial (PCPT)[49]. This trial is a large (approximately 18,000 men) study that was carried out to determine whether the 5 α-reductase inhibitor, finasteride, could reduce the period prevalence of prostate cancer. We will measure the pattern and extent of prostate inflammation and relate these to the presence or absence of prostate cancer.

Urine reflux, chemical and physical trauma. Chemical irritation from urine reflux has been proposed as an aetiological agent for the development of chronic inflammation in the prostate[50]. Although urine contains many chemical compounds that might be toxic

Prostatitis
Technically means 'inflammation of the prostate'. However, it is usually referred to as a clinical syndrome largely characterized by pelvic pain that has several subtypes. Some symptomatic subtypes (I and II) are associated with bacterial infections, others with inflammation but no infection (IIIa), or no inflammation and no infection (IIIb). Type IV consists of chronic inflammation without clinical symptoms.

Expressed prostate fluid
Secretions obtained following prostate massage after digital rectal examination.

Prostate specific antigen
A polypeptide that is expressed at very high levels in prostate epithelial cells, whereas very low levels are detected in normal serum; however, several pathological conditions such as prostate cancer, prostate inflammation and benign prostatic hyperplasia can result in increased serum PSA levels.

Table 1 | **Common somatic genetic and epigenetic changes in prostate cancer**

Gene and gene type	Location	Notes
Tumour-suppressor genes		
CDKN1B	12p13.1–p12	Encodes the cyclin-dependent kinase inhibitor p27. One allele is frequently deleted in primary tumours
NKX3.1	8p21.2	Encodes prostate-restricted homeobox protein that can suppress the growth of prostate epithelial cells. One allele is frequently deleted in primary tumours
PTEN	10q23.31	Encodes phosphatase and tensin homologue, which suppresses cell proliferation and increases apoptosis. One allele is frequently lost in primary tumours. Some mutations are found in primary tumours and more in metastatic lesions
TP53	17p13.1	Has many tumour-suppressor functions, including cell-cycle arrest in response to DNA damage, senescence in response to telomere dysfunction, and the induction of apoptosis. Mutations are uncommon early, but occur in about 50% of advanced or hormone-refractory prostate cancers
Oncogenes		
MYC	8q24	A transcription factor that regulates many target genes involved in cell proliferation, senescence, apoptosis and cell metabolism. Overexpression can directly transform cells. mRNA levels are commonly increased in all disease stages through unknown mechanism(s). Low-level amplification of the MYC locus is common in advanced disease
ERG	21q22.3	Proposed new oncogene for prostate cancer. Fusion transcripts with the 5′ portion of androgen-regulated gene (TMPRSS22) arise from deletion or chromosomal rearrangements commonly found in all disease stages
ETV1–4	7p21.3, 19q13.12, 1q21,-q23, 17q21.31	Encodes ETS-like transcription factors 1–4, which are proposed to be new oncogenes for prostate cancer. Fusion transcripts with the 5′ portion of androgen-regulated gene (TMPRSS22) arise from chromosomal rearrangements commonly found in all disease stages
AR	Xq11–12	Encodes the androgen receptor. Protein is expressed in most prostate cancers, and the locus is amplified or mutated in advanced disease and hormone-refractory cancers
Activation of the enzyme telomerase		Maintains telomere function and contributes to cell immortalization. Activated in most prostate cancers, mechanism of activation may be through MYC activation
Caretaker genes		
GSTP1	11q13	Encodes the enzyme that catalyses the conjugation of reduced glutathione to electrophilic substrates. Functions to detoxify carcinogens. It is inactivated in more than 90% of cancers by somatic hypermethylation of the CpG island within the upstream regulatory region
Telomere dysfunction	Chromosome termini	Contributes to chromosomal instability. Shortened telomeres are found in more than 90% of prostatic intraepithelial neoplasia (PIN) lesions and prostate cancer lesions
Centrosome abnormalities	N/A	Contributes to chromosomal instability. Centrosomes are structurally and numerically abnormal in most prostate carcinomas.
Other somatic changes		
PTGS2, APC, MDR1, EDNRB, RASSF1α, RARβ2	Various	The hypermethylation of CpG islands within upstream regulatory regions occurrs in most primary tumours and metastatic lesions. The functional significance of these changes is not yet known

to prostate epithelium, uric acid itself might be particularly damaging[51]. In support of this, recent work has implicated crystalline uric acid as a 'danger signal' released from dying cells, and it has been shown to directly engage the caspase-1-activating NALP3 (cyropyrin) inflammasome present in cells of the innate immune system (primarily macrophages), resulting in the production of inflammatory cytokines that can increase the influx of other inflammatory cells[52]. In addition, urine reflux of injurious chemicals can function in conjunction with infectious agents to increase prostate inflammation. Another manner by which prostate inflammation might occur is the development of corpora amylacea[53] (FIG 2). Corpora amylacea have been proposed to contribute to prostate inflammation[54], persistent infection[55] and prostate carcinogenesis[56] because they are frequently observed adjacent to damaged epithelium and focal inflammatory infiltrates[54]. In terms of epidemiological research and corpora amylacea, few studies have been conducted, one of which observed a higher proportion of calculi in prostate tissue from patients with prostate cancer compared with patients with BPH[56], whereas others observed no association[57,58]. In support of the concept that 'flushing' of the prostate might be beneficial, several studies have found that increased ejaculation frequency, which might determine the rate of intraluminal corpora amylacea formation and the contact time between chemical agents such as uric acid or other urinary carcinogens and prostatic epithelium[59], is related to decreased prostate cancer incidence (REF. 60 and references therein). Spermatozoa have also been localized to prostate tissues, and the retrograde movement of sperm cells into the prostate has been found in association with inflammation[54,61] and with PIA lesions[61].

Inflammasome
A multiprotein intracytoplasmic complex that activates pro-inflammatory caspases, which then cleave the precursor of interleukin-1β (pro-IL1β) into the active form, leading to a potent inflammatory response.
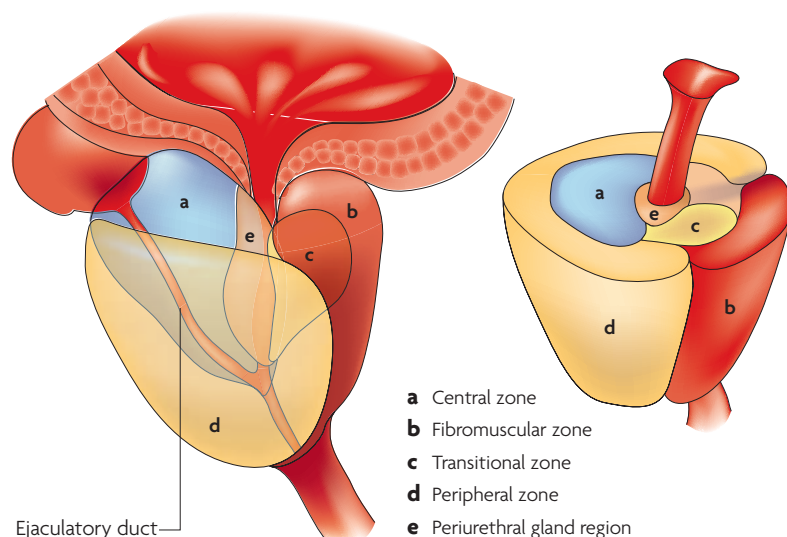
Corpora amylacea
Amorphous small nodules or concretions located in the lumen of benign prostate acini and ducts that accumulate with age.

Dietary factors. Epidemiological studies have revealed a link between prostate cancer incidence and mortality and the consumption of red meat and animal fats[62–64]. One mechanism by which meats might stimulate cancer development could be related to the formation of heterocyclic amines (HCAs)[65]. The exposure of laboratory rats to dietary 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)[66] results in carcinomas of the intestine

in both sexes, in the mammary gland in females and in the prostate in males[65]. Rodent prostates contain four different lobes that do not correspond anatomically to the zones of the human prostate, and PhIP induces cancer only in the ventral lobe of rats[67]. In a recent study we exposed laboratory rats to PhIP and found a similar increase in the mutation frequency in all lobes of the prostate, yet the ventral lobe selectively responded with increased cell proliferation and cell death[68]. Therefore, PhIP functions as both a lobe-specific classical 'tumour initiator' as well as a 'tumour promoter'. We also found that only the ventral lobe showed an increase in stromal mast cells, and stromal and intraepithelial macrophages[68]. After 12 weeks of PhIP exposure, the ventral lobe developed widespread epithelial atrophy; later, PIN and intraductal carcinomas were observed to develop directly from the atrophic epithelium (A.M.D., Y.N. and W.G.N., unpublished observations). Others have recently reported similar findings, in that PhIP treatment was found to induce inflammation and atrophy before inducing PIN and intraductal cancers[69]. Although it is not yet known whether the lobe-specific increase in mast cells and macrophages has a role in the neoplastic process, mast cells have been shown to stimulate cancer formation in several animal models, probably as a result of the release of factors such as tumour necrosis factor-α (TNFα) and various proteases, which might have an important role in tumorigenesis[70–72].

Oestrogens. Another line of research into the causes of prostate inflammation and prostate cancer is the study of oestrogenic exposures in the prostate. Oestrogens are strongly linked to autoimmune processes in women, who are much more predisposed to autoimmune diseases than men. Increased levels of oestrogens, whether from environmental or developmental exposures, have long been linked to the development of prostate cancer[73,74]. Oestrogens affect the growth and development of the prostate, and this occurs through indirect routes on the hypothalamic–pituitary–gonadal axis through prolactin, and also by direct effects mediated by oestrogen receptor-α (ERα), which is expressed primarily in the stroma, and oestrogen receptor-β (ERβ), which is expressed primarily in the epithelium[73–76]. Oestrogens given to neonatal rodents result in an 'imprinted state' or 'developmental oestrogenization' in which there are developmental defects, including a reduction in prostatic growth. This treatment also results in the development of lobe-specific inflammation, hyperplasia and dysplasia or PIN[77,78]. Virtually all of these effects are mediated through ERα[79]. Therefore, it is quite plausible that chronic inflammation in the adult human prostate might reflect an autoimmune reaction caused, at least in part, by oestrogens.

A break of immune tolerance to prostate antigens?
Another potential mechanism of self-perpetuating chronic inflammation in the prostate that could relate to all of the above-mentioned modes of prostate injury is that damaged prostate epithelial cells might release antigens that result in a break of the apparent immune 'tolerance' to the prostate. For example, many prostate

Prostate zones

a Central zone
b Fibromuscular zone
c Transitional zone
d Peripheral zone
e Periurethral gland region

Ejaculatory duct

	Prostate zone		
	Peripheral	Transition	Central
Focal atrophy	High prevalence	Medium-high prevalence	Low prevalence
Acute inflammation	Low prevalence	Low prevalence	Low prevalence
Chronic inflammation	Medium-high prevalence	Low prevalence	Low prevalence
Benign prostatic hyperplasia	None	High prevalence	Low prevalence
High-grade PIN	Medium-high prevalence	Low prevalence	None
Carcinoma	Medium-high prevalence	Low prevalence	None

High prevalence — Low prevalence
Medium-high prevalence — None

Figure 1 | **Zonal predisposition to prostate disease.** Most cancer lesions occur in the peripheral zone of the gland, fewer occur in the transition zone and almost none arise in the central zone. Most benign prostate hyperplasia (BPH) lesions develop in the transition zone, which might enlarge considerably beyond what is shown. The inflammation found in the transition zone is associated with BPH nodules and atrophy, and the latter is often present in and around the BPH nodules. Acute inflammation can be prominent in both the peripheral and transition zones, but is quite variable. The inflammation in the peripheral zone occurs in association with atrophy in most cases. Although carcinoma might involve the central zone, small carcinoma lesions are virtually never found here in isolation, strongly suggesting that prostatic intraepithelial neoplasia (PIN) lesions do not readily progress to carcinoma in this zone. Both small and large carcinomas in the peripheral zone are often found in association with high-grade PIN, whereas carcinoma in the transition zone tends to be of lower grade and is more often associated with atypical adenomatous hyperplasia or adenosis, and less often associated with high-grade PIN. The various patterns of prostate atrophy, some of which frequently merge directly with PIN and at times with small carcinoma lesions, are also much more prevalent in the peripheral zone, with fewer occurring in the transition zone and very few occurring in the central zone. Upper drawings are adapted from an image on Understanding Prostate Cancer website. PIN, prostatic intraepithelial neoplasia.
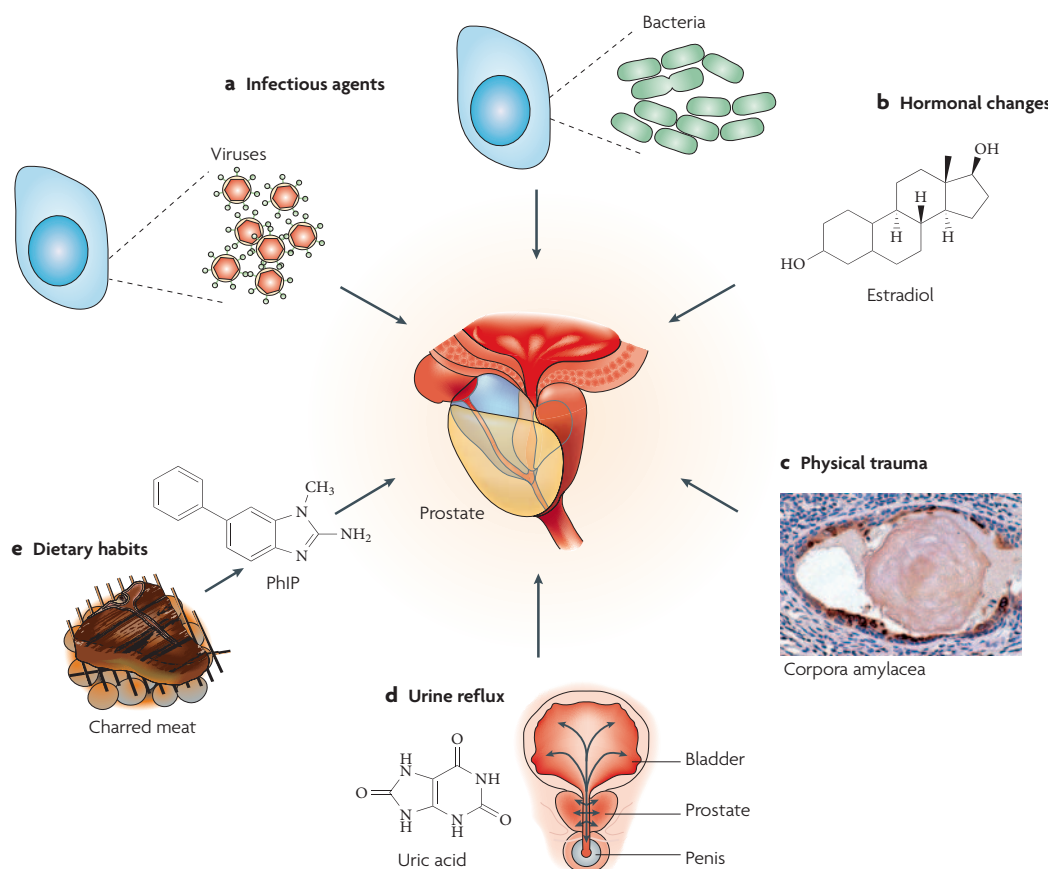
Figure 2 | **Possible causes of prostate inflammation. a** | Infection. Chronic bacterial prostatitis is a rare recurring infection in which pathogenic bacteria are cultured from prostatic fluid. Viruses, fungi, mycobacteria and parasites can also infect the prostate and incite inflammation. The figure represents two prostate cells infected either by bacteria or viruses. **b** | Hormones. Hormonal alterations such as oestrogen exposure at crucial developmental junctures can result in architectural alterations in the prostate that produce an inflammatory response. **c** | Physical trauma. Corpora amylacea can traumatize the prostate on a microscopic level. The figure shows a corpora within a prostatic acinus in which its edges appear to be eroding the epithelium, resulting in an increase in expression of the stress enzyme cyclooxygenase 2 (PTGS2), represented by brown immunostaining. Prostate cell nuclei are visible in violet following haematoxylin staining. **d** | Urine reflux. Urine that travels up back towards the bladder ('retrograde' movement) can penetrate the ducts and acini of the prostate. Some compounds, such as crystalline uric acid, can directly activate innate inflammatory cells. Although these compounds would not be expected to traverse the prostate epithelium, if the epithelium was already damaged this would facilitate the leakage of these compounds into the stromal space where they would readily activate inflammatory cells. **e** | Dietary habits. Ingested carcinogens (for example 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), which derives from charred meat) can reach the prostate through the bloodstream or by urine reflux and cause DNA damage and mutations, and result in an influx of inflammatory cells.

antigens are not expressed until after puberty, when the gland undergoes androgen-stimulated growth and development. This is likely to result in a lack of physiological immune tolerance to these antigens. Therefore, when released during prostate injury, these antigens could prime an immune response resulting in a specific reaction to prostate-restricted antigens. Indeed, a T-cell immune response to PSA in patients with chronic prostatitis has been reported[80].

In summary, many non-infectious mechanisms might lead to prostate epithelial cell and stromal damage. Injured cells are known to signal a 'danger response' that results in acute inflammation. Crystalline uric acid is particularly intriguing in this regard, as it directly interacts with a receptor that is part of a molecular pathway within innate

immune cells that can potently stimulate inflammation. The fact that PhIP induces prostate inflammation and atrophy is also of great interest, as this might link diet to these processes in the prostate carcinogenesis pathway. Continuous exposure to the injurious agent can also set up the prostate for chronic inflammation that can lead to a sustained inflammatory response and cancer. Finally, all of these mechanisms of chronic epithelial injury might also result in a decreased barrier function, that could facilitate the growth of infectious agents that might further increase the inflammatory response, and allow toxic urinary metabolites into the prostatic interstitium, where they could further stimulate an inflammatory reaction. This is certainly an exciting area for continued research into the mechanisms of prostate carcinogenesis.

Heterocyclic amines
Molecules that are produced as a result of cooking meats at high temperatures, and which can be metabolized to biologically active, DNA-damaging agents. 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is the most abundant HCA.

Box 2 | **Somatic genomic alterations in PIA and proliferative atrophy**

In terms of somatic DNA alterations, although normal appearing epithelium (even from cancer patients) does not contain methylated glutathione S-transferase P1 (*GSTP1*) alleles, approximately 6% of focal atrophy lesions contain epithelial cells with methylated *GSTP1* (REF. 25). Another group found mutated p53 (REF. 140) and androgen receptor alleles[141] in post atrophic hyperplasia (a form of focal atrophy), prostatic intraepithelial neoplasia (PIN) and carcinoma, but not in normal prostate tissue — albeit these mutations in post atrophic hyperplasia were apparently non-clonal.

Others have used fluorescent *in situ* hybridization (FISH) to show that there are increases in chromosome 8 centromere signals[142,143], loss of chromosome 8p[143,144] and a gain of chromosome 8q24 in focal atrophy (REF. 143), indicating that chromosomal abnormalities similar to those found in PIN and carcinoma occur in a subset of these atrophic lesions. However, there were no atrophy cases in which clonal alterations were identified. Consistent with this, we recently found no evidence for clonal alterations indicative of chromosome 8 centromeric region gain, 8p loss or 8q24 gain in focal prostate atrophy, and infrequent 8p loss and absent 8q24 gain in PIN[27]. Therefore, the non-clonal mutations and non-clonal chromosomal alterations are probably indicative of genomic damage and/or the emergence of genomic instability in proliferative inflammatory atrophy (PIA) and proliferative atrophy.

In addition to the molecular evidence, other evidence to indicate that PIA and proliferative atrophy represent a field effect in the prostate is that these lesions are often quite extensive in the peripheral zone, often merge directly with high-grade PIN, at times merge directly with small carcinoma lesions and can be directly induced in the rodent prostate by exposures to the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP).

Immunobiology of prostate inflammation

The normal prostate, like all other organs, contains endogenous inflammatory cells consisting of scattered stromal and intraepithelial T and B lymphocytes[81,82], macrophages and mast cells. However, most adult prostate tissues contain increased inflammatory infiltrates, albeit the extent and type of inflammation are variable (for a review, see REF. 83). In terms of the biology of the inflammatory cells and the nature of the immune response in the prostate, most of the work has focused on BPH tissues in comparison with samples from the normal transition zone, and sometimes with carcinoma samples that have occurred in this region. Steiner *et al.* have examined the immunophenotypic and biological properties of chronic inflammatory cells in BPH and normal prostate tissues[84–86]. They have shown that of the increased CD45+ cells (all leukocytes express CD45 and non-leukocytes do not), 70–80% of these are CD3+ T lymphocytes, whereas 10–15% are CD19+ or CD20+ B lymphocytes. Macrophage numbers were also increased in these inflammatory lesions. In terms of the phenotype of the T cells, there is a reversed CD8:CD4 ratio, such that most T cells present in the normal areas expressed CD8, but most T cells in the inflamed areas expressed CD4. In terms of T-cell receptors (TCRs), 90% of the cells represent 'standard' $\alpha\beta$ T cells (which express TCR$\alpha\beta$), with less than 1% representing $\gamma\delta$ T cells. Class II major histocompatability antigen (HLADR), which indicates whether T cells are 'activated' by antigen signalling, is present on approximately 40% of the CD3+ T cells, and many of these T cells expressed CD45RO, indicating that these are 'antigen experienced' T cells[85]. None of the T cells in the normal prostate epithelium showed evidence of either activation or of being antigen experienced T cells.

CD4+ T cell responses can be divided into several different types that are classified according to their cytokine profile. T_H1 cells produce interferon-γ and TNFα, whereas T_H2 cells produce interleukin 4 (IL4), IL5 and IL13. Regulatory T (T_{Reg}) cells, which can suppress adaptive T-cell responses and autoimmunity, are characterized by the expression of CD25 and the transcription factor

FOXP3, and they secrete transforming growth factor-β (TGFβ). In BPH, Marberger's group determined that the T-cell response is complex, in that although T_H0 (T cells that do not express any of the indicated cytokines) and T_H1 cells were predominant in the inflammatory lesions of BPH and in carcinoma, some features of a T_H2 response were also present. Unfortunately, at this point similar experiments have not been performed in the other zones of the prostate, or in areas of focal atrophy or PIN of the peripheral zone. The need for further understanding in this area is crucial, as is illustrated by the findings that microbially-driven inflammation can lead to colon cancer in mice, and that the prior transfer of T_{Reg} cells that express CD4 and CD25 prevents the inflammatory response that leads to colon cancer in these animals[87]. Recently Miller *et al.*[88], have shown that CD4+ and CD25+ T cells, with properties of T_{Reg} cells including the expression of the FOXP3 protein, are present in increased numbers in clinically localized prostate cancer tissues, compared with normal prostate tissues. Exciting new data from several groups suggest the importance of a new subset of CD4-effector T cells known as T_H17 cells, which develop through distinct cytokine signals (especially IL23) with respect to those involved in T_H1 and T_H2 responses, and are characterized by the production of IL17 (REF. 89). These cells are required for inflammation in arthritis and encephalitis models[89], and IL23 is required for skin cancer formation in response to carcinogen exposure in mice[90]. A potential role for T_H17 cells in prostatic inflammation had already been demonstrated by Steiner *et al.* before the T_H17 cell lineage had been recognized as being as distinct. They showed that activated T cells in BPH tissue and in prostate cancer express high levels of IL17 (REF. 85). Further work to more fully elucidate the phenotypic and biological properties of all T-cell subsets in the prostate is required before we can understand the significance of acquired cell-mediated immunity in prostate carcinogenesis. Methods such as the quantitative image analysis of immunohistochemically stained inflammatory cell subsets, as well as flow cytometry for these subsets using tissues isolated from histologically defined areas, will be crucial to obtain such data.

FOXP3
A nuclear transcription factor that is expressed specifically in regulatory T cells.

Box 3 | Molecular pathways altered in PIA and proliferative atrophy

In terms of molecular modes of action, p27 functions as an inhibitor of cell-cycle progression by inhibiting the activity of cyclin–cyclin dependent kinase complexes in the nucleus. Interestingly, p27 levels are generally reduced but not absent in human proliferative inflammatory atrophy (PIA), prostatic intraepithelial neoplasia (PIN) and prostate cancer[23,145–147]. The fact that p27 levels are not lost entirely (or biallelically inactivated by mutations) in cancer might be explained by recent findings that indicate that cytoplasmic p27 levels, which are increased by signalling through the MET receptor tyrosine kinase, are required for cell migration in response to hepatocyte growth factor signalling through MET and in response to increased cyclin D1 levels[148]. Therefore, although high levels of nuclear p27 can prevent cell-cycle progression, cytoplasmic p27 might be required for optimal tumour cell motility, which is a key feature of malignant transformation and tissue repair.

Phosphatase and tensin homologue (PTEN) is a dual protein and lipid phosphatase that is responsible for the dephosphorylation and inactivation of phosphatidylinositol 3,4,5-trisphosphate (PIP3), a second messenger produced after the activation of PIP3 kinase in response to the ligation of several growth factor receptors, including the insulin-like growth factor 1 receptor (IGF1R)[149]. PIP3 is required for the activation of the protein kinase AKT. AKT activation results in the inhibition of apoptosis and/or increased cell proliferation through several different effector mechanisms, such as the activation of mammalian target of rapamycin (mTOR) and S6 kinase.

NKX3.1 is a prostate-restricted homeodomain protein encoded within a region of chromosome 8p21 that often contains single copy deletions in prostate cancer[150]. In addition to suppressing the growth of prostate cells, decreased NKX3.1 protein levels result in increased oxidative DNA damage[151].

PIA and proliferative atrophy also show increased BCL2 protein expression[18], a gene product that is a potent suppressor of apoptosis. Other gene products that are increased in PIA and proliferative atrophy include those that are induced by oxidant and electrophilic stress, or by signals associated with cell activation and proliferation, including glutathione S-transferase P1 (GSTP1), GSTA1, cyclooxygenase 2 (PTGS2) and p16 (REFS 18,152,153). The fact that these stressed cells are undergoing tissue repair is supported by the finding that several proteins known to be involved in tissue repair and cell motility, such as MET[23] and HAI1 (REF. 154), have increased expression in PIA and proliferative atrophy.

Inflammatory genes and prostate cancer risk

Through a variety of approaches, including family and twin studies and segregation analyses, an important role for an inherited component of prostate cancer risk has been documented (recently reviewed by Schaid[91]). These studies have set the stage for efforts to identify prostate cancer susceptibility genes using linkage analysis and, more recently, association-based approaches. Despite strong evidence for a genetic component to prostate cancer risk, few reliable genetic risk factors for prostate cancer have been identified. In this section we will focus on a relatively new area of investigation in this field: the possibility that allelic variants of genes involved in innate and acquired immunity play an important part in determining inherited prostate cancer risk. If chronic inflammation is indeed an important aetiological factor for prostate cancer, then allelic variants of the genes involved in inflammatory pathways are logical candidates for genetic determinants of prostate cancer risk. As a result of space limitations, we can only review what we consider the most well-studied examples to date.

RNASEL and MSR1. Following up genomic regions of interest identified by linkage studies of prostate cancer families, two genes involved in innate immunity unexpectedly emerged as candidate prostate cancer susceptibility genes. Inactivating mutations (E265X and M1I) in ribonuclease L (*RNASEL*) segregate with prostate cancer in two prostate cancer families: E265X with one of European descent and M1I with a family of African descent[92,93]. *RNASEL*, which is located at 1q25, is a component of the innate immune system that is required for the antiviral and antiproliferative roles of interferons[94,95]. Lymphoblasts from carriers of either one of the mutations mentioned above were found to be deficient in enzymatic RNase activity, although, other than prostate cancer, additional phenotypic manifestations were not obvious. Subsequent studies examining the role of *RNASEL* as a prostate cancer susceptibility gene have provided mixed evidence, some confirmatory[91] and others not[91,96–99]. Although the association of this infection with prostate cancer development has yet to be shown[40], carriers of a common, hypomorphic allele of *RNASEL* (*R463Q*) were found to be at risk for prostatic infection by a new γ-retrovirus. Interestingly, when RNASEL is activated in cells by its cognate interferon-inducible ligands, 2′,5′-linked oligoadenylates, mRNA species are consistently induced, one of which is encoded by the (macrophage-inhibitory cytokine 1) *MIC1* gene, which is another prostate cancer susceptibility locus described below[100].

The analysis of candidate genes in a different region of linkage (8p22) in prostate cancer families revealed several recurring, inactivating mutations in macrophage scavenger receptor 1 (*MSR1*)[101]. The *MSR1* gene encodes a homotrimeric class A 'scavenger receptor', with expression largely restricted to macrophages. This receptor is capable of binding many ligands, including modified lipoproteins and both Gram-negative and Gram-positive bacteria. Mice with experimentally inactivated *Msr1* are more susceptible to various types of bacterial infection[102,103], although recent evidence suggests an anti-inflammatory role for this receptor, at least after exposure to certain pathogens[104,105]. *MSR1* mutant alleles, *R293X* and *H441R*, which are found in several different prostate cancer families, code for proteins that can no longer bind bacteria or modified LDL (C.M. Ewing and W.B.I., unpublished observations). Despite the initial findings of an increased frequency of *MSR1* mutations in men with prostate cancer, as with *RNASEL*, follow-up studies published on the possible role of *MSR1* variants and prostate cancer risk have yielded inconsistent results[98,101,106–111]. A recent meta analysis suggests that *MSR1* mutations might have a more reproducible effect on prostate cancer risk in African Americans[112]. Although these results do not indicate that these genes are major prostate cancer loci, they are consistent with these genes being able to modify prostate cancer risk, possibly in combination with particular environmental exposures — in this case, certain pathogens.

Toll-like receptors. The Cancer Prostate Sweden Study (CAPS) is a case–control study of prostate cancer in northern Sweden. The relative genetic homogeneity of the Swedish population and the large size of the CAPS study make it an ideal platform to identify genetic variants associated with prostate cancer risk. Studying cases and controls in CAPS over the past 3 years has led to the identification of several genes in inflammation-related pathways, including *MIC1*, interleukin 1 receptor antagonist (*IL1RN*) and members of the toll-like receptor (TLR) family, with allelic variants associated with prostate cancer risk.

As key players in innate immunity to pathogens, TLRs recognize pathogen-associated molecular patterns (PAMPs)[113]. The engagement of TLRs results in the production of various pro-inflammatory cytokines, chemokines and effector molecules, such as reactive oxygen and nitrogen intermediates, as well as upregulation of the expression of co-stimulatory CD86 and CD80 and major histocompatability complex II (MHC II) molecules, which facilitate adaptive immune responses. Ten members of the human TLR family have been identified, and for most of these, specific classes of ligands, typically microbial components or surrogates thereof, have been identified and characterized. Recently, sequence variants in several TLR genes have been linked to prostate cancer risk, including *TLR4* and the *TLR1–6–10* gene cluster[114,115].

Ligands that are recognized by TLR4 include Gram-negative bacterial products, including lipopolysaccharide[116], and human heat shock protein 60 (HSP60)[117]. In the CAPS study[114], a single nucleotide polymorphism (SNP) in the 3′ UTR region of *TLR4* (11381G/C) was found to be associated with prostate cancer risk. Carriers of the GC or CC genotypes of this SNP had a 26% increased risk of prostate cancer, and a 39% increased risk of early-onset prostate cancer (before the age of 65 years), compared with men with the wild-type GG genotype.

In a follow up study of a North American population, homozygosity for variant alleles of eight SNPs in *TLR4* (REF. 118) was associated with a statistically significantly lower risk of prostate cancer; however, the *TLR4_15844* polymorphism, which corresponds to 11381G/C implicated in the CAPS population, was not found to be associated with prostate cancer. Therefore, although both published studies of this gene indicate that genetic variants of *TLR4* have a role in the development of prostate cancer, the specific variants responsible for this effect might vary across different populations.

The *TLR1–6–10* cluster maps to 4p14, and encodes proteins that have a high degree of homology in their overall amino-acid sequences[119]. TLR6 and TLR1 recognize diacylated lipoprotein and triacylated lipoprotein as ligands, respectively[120,121]. However, no specific ligand has been identified for TLR10. The TLR1 and TLR6 proteins each form heterodimers with TLR2 to establish a combinational repertoire that distinguishes a large number of PAMPs[120,122,123].

A study of the *TLR1–6–10* cluster in prostate cancer patients in CAPS identified an association of sequence variants in *TLR1–6–10* with prostate cancer risk[114,115]. The allele frequencies of 11 of the 17 SNPs examined in this gene cluster were significantly different between case and control subjects (P = 0.04–0.001), with odds ratios for variant allele carriers (homozygous or heterozygous) compared with wild-type allele carriers ranging from 1.20 (95% CI = 1.00–1.43) to 1.38 (95% CI = 1.12–1.70). Although further studies are necessary to understand the biological consequences of the risk variants in both *TLR4* and the *TLR1–6–10* cluster, the observation of prostate cancer risk associated with polymorphisms in this family of genes, which is so intimately related to innate immunity, indicates that inflammation-related processes are important in prostate cancer development.

MIC1. MIC1 is a member of the transforming growth factor-β (TGFβ) superfamily, and is thought to have an important role in inflammation by regulating macrophage activity. In a study of 1,383 patients with prostate cancer and 780 control subjects in CAPS, a significant

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Odds ratio
The odds ratio is a way of comparing whether the probability of a certain event is the same for two groups, and is calculated using a 2×2 table. An odds ratio of one implies that an event is equally likely in both groups. An odds ratio greater than one implies that an event is more likely in the first group. An odds ratio less than one implies that the event is less likely in the first group.

Longitudinal study
A study in which repeated observations of a set of subjects are made over time with respect to one or more study variables.

Box 4 | **The epidemiology of STIs and prostate cancer**

Epidemiological studies of sexually transmitted infections (STIs) and prostate cancer initially focused on gonorrhea and syphilis. Dennis and Dawson[155] combined the results of these studies and estimated summary odds ratios (ORs) of 1.4 for the development of prostate cancer in patients with a history of any STI, 2.3 for a history of syphilis and 1.4 for a history of gonorrhea. Similar estimates were also calculated in a subsequent meta-analysis[156]. Another recent case–control study reported that a history of both gonorrhea and more than 25 previous sexual partners were associated with an increased risk of prostate cancer[46]. The significance of many of these studies is limited, as most were small case–control designs that may have been susceptible to selection, recall and interviewer bias. In terms of other STIs, we recently observed that men who carried antibodies against *Trichomonas vaginalis* had a higher risk of prostate cancer than men who did not, and the association was stronger in men who rarely used aspirin[157].

In another inquiry we conducted a longitudinal study of young (median age <31) STI clinic patients by measuring serum prostate specific antigen (PSA) as a marker of prostate infection and damage. Men with an STI were more likely to have a ≥40% increase in serum PSA than men without an STI diagnosis (32% versus 2%, P <0.01)[158]. Increases in PSA levels were strongly suggestive of direct prostate involvement by the infectious agent, with resultant epithelial cell damage (either due to the organism itself or the inflammatory response to the organism) resulting in the release of PSA into the blood stream. As only about 32% of patients with acute STIs showed increased levels of PSA, it is apparent that either these agents do not always infect the prostate, or they do not illicit a strong inflammatory response that damages prostate tissues, or rapid antibiotic treatments prevent full-blown prostate involvement.

In terms of viral STIs and prostate cancer, Strickler and Goedert[36] concluded that those studied to date are unlikely to contribute to prostate carcinogenesis, although they did suggest the possibility of a causal relationship between an as-yet unresearched and unidentified infectious agent and prostate cancer. Urisman and colleagues[40] recently identified a novel γ-retrovirus in prostate tissues primarily from patients with germline *RNASEL* mutations. This intriguing finding is a proof of concept that specific infectious agents might persist in the prostate as a result of heritable changes in genes responsible for the clearance of these agents.
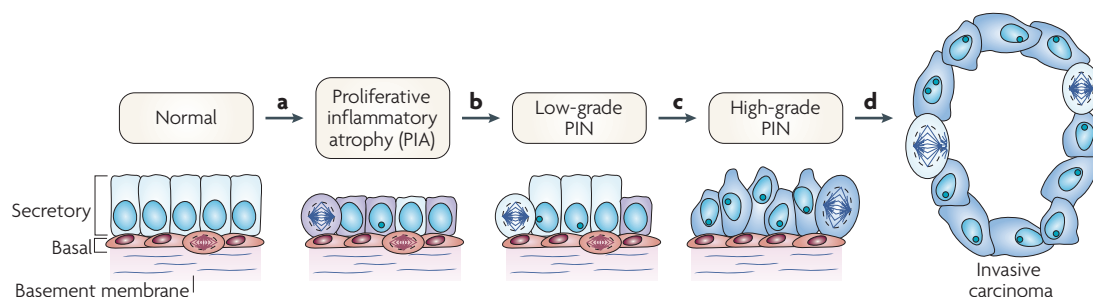
Figure 3 | **Cellular and molecular model of early prostate neoplasia progression. a** | This stage is characterized by the infiltration of lymphocytes, macrophages and neutrophils (caused either by repeated infections, dietary factors and/or by the onset of autoimmunity); phagocytes release reactive oxygen and nitrogen species causing DNA damage, cell injury and cell death, which trigger the onset of epithelial cell regeneration. The morphological manifestation of the cellular injury is focal prostate atrophy, which is proposed to signify the 'field effect' in the prostate. The downregulation of p27, NKX3.1 and phosphatase and tensin homologue (PTEN) proteins in luminal cells stimulates cell-cycle progression. Stress-response genes are induced (such as glutathione S-transferase P1 (*GSTP1*), *GSTA1* and cyclooxygenase 2 (*PTGS2*)). **b** | The subsequent silencing of *GSTP1* through promoter methylation in subsets of cells further facilitates oxidant-mediated telomere shortening. **c** | Cells carrying methylated *GSTP1* alleles and short telomeres have dysfunctional telomeres and are more likely to bypass the senescence checkpoints. This favours the onset of genetic instability and the consequent accumulation of genetic changes (for example loss of heterozygosity on 8p21,6q or gain of function on 8q24,17q). **d** | The continued proliferation of genetically unstable luminal cells and the further accumulation of genomic changes, such as gene rearrangements leading to *TMPRSS2–ETS* family member gene fusions, lead to progression towards invasive carcinomas. PIN, prostatic intraepithelial neoplasia.

difference ($P = 0.006$) in genotype frequency was observed for the non-synonymous change H6D between patients and controls[124]. Carriers of the GC genotype, which results in the H6D change, had a lower risk of sporadic prostate cancer (OR = 0.80, 95% CI = 0.66–0.97) and of familial prostate cancer (OR = 0.61, 95% CI = 0.42–0.89) than the CC genotype carriers. In the study population, the proportion of prostate cancer cases attributable to the CC genotype was 7.2% for sporadic cancer and 19.2% for familial cancer.

IL1RN. The protein product of the *IL1RN* gene belongs to the interleukin 1 cytokine family of proteins. Its primary function is as an inhibitor of the proinflammatory IL1α and IL1β. Lindmark *et al.* examined four haplotype-tagging SNPs (htSNPs) across the *IL1RN* gene in samples from patients with prostate cancer[125]. The most common haplotype (ATGC) was observed at a significantly higher frequency in the cases (38.7%) compared with the controls (33.5%) ($P = 0.009$). Carriers of the homozygous ATCG haplotype had significantly increased risk (OR = 1.6, 95% CI = 1.2–2.2). Furthermore, the association of this haplotype was even stronger among patients with advanced disease compared with controls[125].

Other inflammatory-related genes

Many other genes in inflammatory pathways have been examined recently for a link to prostate cancer, generally with mixed results. For example, although McCarron *et al.* previously reported an association between certain alleles of *IL10* and *IL8* and prostate cancer[126], Michaud *et al.* recently reported a lack of association of polymorphisms in the *IL1β, IL6, IL8* and *IL10* and prostate cancer in a case–control study of the Prostate, Lung, Colorectal,

and Ovarian Cancer screening trial[127]. Further work is necessary to either confirm or refute the hypothesis that variants in genes associated with inflammation affect prostate cancer risk, and if confirmed, to understand the mechanisms that link allelic variation in inflammation genes and prostate cancer.

SNPs and the inflammatory pathway

In a more global genome-wide approach, Zheng *et al.*[128] proposed that sequence variants in many other genes in the inflammatory pathway might be associated with prostate cancer. They evaluated 9,275 SNPs in 1,086 genes of the inflammation pathway among 200 familial cases and 200 unaffected controls selected from the CAPS study population. They found that more than the expected numbers of SNPs were significant at a nominal P value of 0.01, 0.05 and 0.1, providing overall support for the hypothesis. A small subset of significant SNPs (N = 26) were selected and genotyped in an independent sample of ~1,900 members of the CAPS population. Among the 26 SNPs, six were significantly associated with prostate cancer risk ($P \leq 0.05$). These results are consistent with the idea that variation in many genes in inflammatory pathways might affect the likelihood of developing prostate cancer.

Ideally, one would prefer to correlate the presence of specific genetic polymorphisms with the pattern and extent of intraprostatic inflammation, yet in all of the studies reported above the status of the prostate in men in terms of presence, pattern and extent of inflammation is unknown. Future studies that address these issues will be crucial in evaluating the biological effects of various polymorphisms in inflammatory pathway genes.

The 'injury and regeneration' hypothesis

Our current working model (FIG. 3) suggests that repeated bouts of injury (and cell death) to the prostate epithelium occur, either as a result of oxidant and/or nitrosative damage from inflammatory cells in response to pathogens or autoimmune disease, from direct injury from circulating carcinogens and/or toxins derived from the diet or from urine that has refluxed into the prostate. The morphological manifestation of this injury is focal atrophy or PIA, which we postulate to be a signature of the 'field effect' of prostate carcinogenesis. The biological manifestations are an increase in proliferation and a massive increase in epithelial cells that possess a phenotype intermediate between basal cells and mature luminal cells[5,6,23]. In a small subset of cells, perhaps cells with an intermediate phenotype that contain at least some 'stem cell' properties, somatic genome alterations occur, such as cytosine methylation within the CpG island of the *GSTP1* gene and telomere shortening. Both of these molecular changes can decrease the 'caretaker' phenotype and increase genetic instability that might then initiate high-grade PIN and early prostate cancer formation. In the setting of ongoing inflammatory and dietary insults in cells with compromised caretaker functions, additional changes such as gene rearrangements resulting in the activation of the ETS family of oncogenic transcription factors, the activation of *MYC* expression and the loss of tumour-suppressor genes such as *PTEN*, *NKX3.1* and *CDKN1B* occur that drive tumour progression**.**

Future directions

We reviewed evidence that in men with an underlying genetic predisposition, prostate cancer might be caused by inflammation possibly coupled with dietary factors. However, additional work needs to be done to determine whether the mechanisms proposed are correct. First, we need an improved ability to diagnose and define clinical 'prostatitis'. Second, we need studies that quantify asymptomatic inflammation in the prostate to determine the relationship between the development of prostatic inflammation and the following: age, genotype, response to specific infectious organisms, and diet. We need an improved understanding of the types of inflammatory cells and their biological properties in the normal prostate and in the various lesions such as PIA, BPH, PIN and carcinoma. Another potential avenue for future studies is to couple improvements in imaging of the prostate, including new strategies to image inflammation and atrophy, to studies aimed at quantifying various types of inflammation in prostate biopsy specimens and quantitative analyses of cytokine profiles and inflammatory cell types in prostate fluid. These studies should also be performed in conjunction with experiments designed to identify specific infectious organisms. It will be crucial in these studies to have both the genetic information and the dietary and medical history data to correlate with the immunobiological data. Improvements in our understanding of the key molecular genetic and epigenetic events that

drive prostate carcinogenesis, and the identification of the precise cell types involved (that is, whether prostate epithelial stem cells or their progeny are directly transformed) need to be applied to presumed precursor lesions to define precisely the order of events in the development of early prostate cancer. As animal models of prostate cancer continue to be developed that mimic the human disease, such as those that activate *MYC* or inactivate *PTEN*, *CDKN1B* or *NKX3.1* (REFS 28,129), strategies for determining whether infectious agents and/or specific activated inflammatory cells are required for prostate carcinogenesis also need to be developed. Examples of such studies include crossing mice that are genetically engineered to develop prostate cancer with mice that lack specific subsets of cells of the innate and adaptive immune systems, to determine the contribution of such cells to the transformation process. In other studies, one can target inflammation to the prostate by using transgenic technologies to overexpress chemokines and/or cytokines that attract inflammatory cells to the prostate or that will activate inflammatory cells that are already resident in the prostate. As rodents are quite resistant to prostate cancer development, these studies would be potentially more informative if they were carried out on genetically altered animals already prone to developing early neoplastic lesions in the prostate.

Inflammation is a very complex process, which involves hundreds of genes. Therefore, there are many genes in the inflammatory pathways that might contribute to the development of prostate cancer. Whereas many genes in the inflammatory pathway have been shown to harbour sequence variants that may or may not be associated with increased risk of prostate cancer, larger studies in different study populations are needed to confirm and more thoroughly characterize the associations discussed above. Traditional association tests that examine one gene at a time remain valuable approaches, but they are fast being supplanted by new approaches that provide an efficient and economically feasible way to study virtually all of the genes in the whole pathway. Technologies using bead-based or chip-based arrays allow for the rapid examination of thousands of SNPs among hundreds of genes, and even genome-wide searches assessing all genes. Such approaches will provide a comprehensive evaluation of genes in inflammatory pathways, and will provide an appropriate perspective of the importance of genes in these pathways in the context of all known cellular pathways. Although the data obtained so far using genome-wide scans are promising, the challenges of such studies are significant, as many associations are expected to occur by chance. Only when specific associations are validated in multiple large cohorts will the scientific community have confidence in the purported findings from such studies. Although it is currently unknown whether or not 8q24 is related to inflammatory pathways, one very recent example of a success story stems from a set of independent studies that implicated this chromosome region in prostate cancer occurring in both families and in sporadic cases[130,131].

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DATABASES
The following terms in this article are linked online to:
Entrez Gene:
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CDKN1B | CD3 | CD4 | CD8 | CD19 | CD20 | CD45 | CD80 | CD86 | ERα | ERβ | FOXP3 | GSTP1 | HSP60 | IL1RN | interferon-γ | IL4 | IL5 | IL13 | IL17 | IL23 | MIC1 | MSR1 | MYC | NKX3.1 | PTEN | RNASEL | TLR1 | TLR4 | TLR6 | TLR10 | TNFα |

FURTHER INFORMATION
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Understanding Prostate Cancer: http://studentweb.usq.edu.au/home/q9210374/site/index.html
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Purpose

PhIP is the most abundantly contatined dietary carcinogen heterocyclic amine in charred meat. It is reported that PhIP induce prostate cancer in the rat ventral prostate. In this prostate cancer model, increase in Mutation Frequency (MF) and spontaneous inflammation in the ventral prostate precede the development of prostate cancer. Recently, the anticarcinogenic effects of selective COX-2 inhibitors not through the anti-inflammatory effects are reported. Our purpose is to investigate if selective COX-2 inhibitor celecoxib has an effect to the early phase of the carcinogenic process in the PhIP induced rat prostate cancer.

Method

We used Fisher344 BigBlue^{TM} rats, which enable us to calculate mutation frequencies (MFs) by Big Blue assay. Forty rats were divided into 4 groups; 1) normal diet, 2) normal diet+celecoxib 1500ppm, 3) PhIP 400ppm, 4) PhIP 400ppm+celecoxib 1500ppm. After 12 weeks, mutation frequencies were calculated.

Results

There were no differences in the incidence of acute or chronic prostatitis in each group. Mutation frequency in the ventral prostate was significantly increased in PhIP treated group but this increase was significantly reduced when treated with celecoxib.

Conclusion

Celecoxib reduced the PhIP induced increase in the mutation frequencies. Although further investigation is needed, it is suggested that celecoxib could be a preventive drug for prostate cancer through its anti-mutagenic effect.

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Paradoxical downregulation of C-MYC and upregulation of NKX3.1 proteins during invasion in the Lo-MYC mouse prostate

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Abstract:
Introduction and Objective: *C-MYC* has been implicated in human prostate cancer and targeted overexpression of *C-MYC* in the mouse results in prostatic intraepithelial neoplasia (PIN) and invasive adenocarcinoma (CaP) (Cancer Cell. 4:223-238, 2003). However, the onset and dynamics of C-MYC protein expression, and its relation to morphological transformation, the expression of other prostate tumor suppressor proteins, early invasion and the development of inflammation have not been addressed. *Methods:* Lo-MYC and wild type mice were sacrificed at various ages up to 52 weeks. The prostate lobes were dissected and processed for histological and immunohistochemical analysis for C-MYC, androgen receptor (AR), Nkx 3.1, p27, Ki67, cleaved caspase 3, F4/80, CD3, CD45, p63 and smooth muscle actin. *Results:* All mice showed PIN at 4 weeks of age with no invasion until 52 weeks. The morphological alterations in PIN included an increase in cell and nuclear size, nucleolar enlargement, hyperchromasia, increased mitoses and apoptotic bodies. Detection of C-MYC protein coincided with morphological alterations, suggesting that C-MYC is sufficient for transformation. All mice showed microinvasive CaP by 52 weeks, and both the level of C-MYC protein and the degree of cytological atypia were decreased upon early invasion, with recovery of C-MYC protein and nuclear atypia upon enlargement of the invasive tumor. Gland formation and AR expression were maintained during early invasion. The expression of Nkx 3.1 was inverse to that of C-MYC. Decreased p27 and increased Ki67 and cleaved caspase 3 were found in PIN and CaP. Inflammatory cells, consisting of mostly of lymphocytes and F4/80 positive macrophages increased in an around the lesions as they progressed. *Conclusions:* We verified that Lo-MYC mice develop PIN and early CaP that resemble the human disease. Our new data show: (i) C-MYC appears to be sufficient to morphologically transform prostate cells into PIN; (ii) C-MYC protein is decreased and Nkx3.1 protein is increased transiently during invasion; (iii) inflammatory cell infiltrates accompany the development and progression of PIN to CaP. These results validate the Lo-MYC mouse to model early human CaP and reveal complex dynamics of C-MYC and Nkx3.1 protein expression and inflammation during disease progression.
Author Disclosure Information: T. Iwata, None; **A.M. DeMarzo**, None.

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Glutathione S-transferase pi (GSTP1) deficiency accelerates prostate carcinogenesis in the Lo-MYC mouse

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Abstract:
Introduction and Objective: C-MYC has been implicated in human prostate cancer and targeted overexpression of human C-MYC in the mouse results in prostatic intraepithelial neoplasia (PIN) and invasive adenocarcinoma (CaP) (Cancer Cell. 4:223-238, 2003). GSTP1, encoding glutathione *S*-transferase-pi, has been proposed to be a caretaker gene, protecting cells against genome damage mediated by oxidants and electrophiles. The vast majority of human PIN and CaP fail to express GSTP1 as a result of silencing by CpG island hypermethylation of its promoter. In this study, we crossed Lo-MYC mice to GSTP1 -/- mice in order to investigate the effects of GSTP1 deficiency in prostate carcinogenesis. Methods: Lo-MYC mice were crossed to GSTP1 -/-. These mice were sacrificed at various ages up to 7 months. The prostate lobes were dissected and processed for histological and immunohistochemical analysis for C-MYC, androgen receptor (AR), NKX 3.1, p27, Ki67, cleaved caspase 3, and smooth muscle actin. Results: All GSTP1 -/- showed PIN at 4 weeks of age like the standard Lo-MYC mice. All GSTP1 -/- showed more advanced PIN lesions, characterized by cribriform formation, and an altered cytological appearance reminiscent of invasive adenocarcinoma, than Lo-MYC by 3 months. Both GSTP1 -/- and GSTP1 +/- developed microinvasive adenocarcinomas by 6 months, while Lo-MYC didn't develop invasive adenocarcinomas until 12 months. Conclusions: Inactivation of GSTP1 in mice accelerates MYC-induced prostate carcinogenesis. This study is the first to provide direct evidence that GSTP1 can function as a tumor suppressor in the prostate.

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2: **Iwata T**, Meeker AK, Smearman E, De Marzo AM : The

Telomere shortening in prostatic atrophy lesions. The annual meeting of United States and Canadian Academy of Pathology, 2008

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